IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Remacle et al.
Appl. No.	:	09/816,763
Filed	:	March 23, 2001
For	:	METHOD AND KIT FOR THE SCREENING, THE DETECTION AND/OR THE QUANTIFICATION OF TRANSCRIPTIONAL FACTORS
Examiner	:	Kim, Young J.
Group Art Unit	:	1637

DECLARATION UNDER 37 C.F.R §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. This Declaration is being submitted to demonstrate that the claimed invention unexpectedly provides the ability to rapidly determine which of one or more activated transcription(al) factors(s) is/are present in a cell or cell lysate with high sensitivity and specificity.

2. I am an inventor on the above-identified patent application and am familiar with the specification and prosecution history.

3. I have extensive experience in the field of the claimed invention as indicated in the attached Curriculum Vitae provided herewith as Exhibit A.

4. The claimed invention provides accurate screening and/or quantification method of one or more activated transcription factor(s) present in a cell or cell lysate. In order to provide specificity, the claimed invention provides: a double-stranded DNA sequence comprising a

specific sequence which is specifically recognized by activated transcription factor(s) and means for identifying and/or quantifying a signal specific for the binding of said activated transcription factor(s) upon said double-stranded DNA sequence(s), for example through the use of antibodies specific for the activated transcription factor(s). In order to provide particular technical features related to the invention as explained below, the double-stranded DNA sequence is linked to a spacer which comprises a double-stranded nucleic acid part which is between about 50 and about 250 base pairs and which is not present in the cell containing the activated transcription factor(s) to assay.

5. The present invention was designed to overcome a problem in prior art methods for the screening and/or quantification of activated transcription factor(s) present in a cell or cell lysate. The process of screening should not be overly time consuming. The problem of identifying activated transcription factors is that one has to have high sensitivity to be able to detect low amounts of one or more activated transcription factors in a cell or cell lysate, which represents only a small fraction of the total amount of proteins in presence, and a high specificity to be able to distinguish among activated and non activated transcription factors present in the sample, but also to only bind and detect the target transcription factor(s). Traditionally, transcription factor activity has been studied using either Electrophoretic Mobility Shift Assay (EMSA), immunoblotting or reporter gene assays. The problem in the prior art methods is that they are quite time-consuming and at best, provide only semi-quantitative results. In order to obtain good sensitivity of transcription factors detection, the EMSA method proposes the use of short radioactive double-stranded oligonucleotide probes in the range of 20 bp, containing the specific sequence of transcription factor binding. These radioactive probes are incubated in solution with cell extracts and if the transcription factors are present in the cell extract, they bind to their specific sequence. Samples are then resolved by native polyacrylamide gel electrophoresis followed by autoradiography. A retarded band, corresponding to transcription factor/probe complexes appears, in addition to the fast migrating band corresponding to the free probe. Under those basal conditions, however, only limited specificity is reached, as multiple transcription factors can bind a same specific sequence, i.e. if they belong to the same binding family (examplified by the CREB family: Shaywitz and Greenberg (1999) *Annu.Rev. Biochem.* **68**:821-

861, see attached). In addition, many transcription factors can bind DNA without being activated, their activation relies on structural modifications occuring after the binding (Shaywitz and Greenberg (1999) *Annu.Rev. Biochem.* **68**: 821-861). To identify which transcription factor, and under which form, actually formed the complex of the retarded band, EMSA uses supershift experiments. An antibody which specifically recognizes the target transcription factor, and possibly its activated form, is added to the mix prior to electrophoresis. A slow-migrating, 'supershifted' band appears if a complex between the transcription factor/DNA/antibody did form. Those skilled in the art acknowledge that supershifted bands are hardly observable and difficult to quantify.

6. Therefore, EMSA is poorly specific, poorly sensitive, time consuming and does not allow the handling of a large number of samples. Thus the method is difficult to adapt to automation and is not suited for screening. In addition, it is based on the use of ^{32}P radioactive probes. If the probe has a length corresponding to the exact sequence of the binding site for the transcription factor (typically 4-8 nucleotides), it is not sufficient to obtain a sensitive detection, and additional bases are necessary to allow the formation of stable complexes between the factor and the probe. On the other hand, if the radioactive probe is too long, then the method has low specificity due to the undesired cross-binding of transcription factors present in the sample to sequences adjacent, or even overlapping, the specific binding site (4-8 nucleotides).

7. To address the specificity issue, prior art assays have typically used short DNA sequences and to address the sensitivity issue, they have used radioactive probes which are incubated in solution with the transcription factors. However, theses options necessarily invoke one of the disadvantages raised above.

8. We unexpectedly found that if a double-stranded DNA sequence is connected to the surface of the solid support via a spacer containing a double-stranded nucleic acid part which is between about 50 and about 250 base pairs in length, the detection of small amounts of activated transcription factors gains in sensitivity without loosing high specificity. We also found that using a spacer whose nucleic acid part is not present in the cell containing the activated transcription factors to assay allowed gaining in specificity without loosing the sensitivity.

9. Keeping the detection sensitivity together with the specificity for detection of one or more activated transcription factors was an unexpected result of the present invention.

10. The ability of the claimed invention to measure the activity of transcription factors (simple and quantitative assay) and to rapidly yield results is extremely valuable in commercial applications. The kit using the method of the present invention is currently commercialized by Eppendorf AG. The product is referred to as TF Chip MAPK, which is able to simultaneously quantify eight activated transcription factors (AP1 (c-Jun), ATF2, c-Myc, Elk-1, MEF2, NFATc1, p53 and STAT1) on a micro-array.

11. In order to provide high sensitivity, the claimed invention utilizes a relatively long double-stranded capture probe immobilized on a solid support, comprising a spacer which comprises a double-stranded DNA nucleotide sequence of between about 50 and about 250 base pairs in length. The use of a long spacer allows increasing the sensitivity of the method on a solid support for which the steric hindrance is higher than a reaction performed in solution. Also, there is no need to label the capture probe like in the EMSA and the cell extract can be directly contacted with the insoluble solid support without any further treatment. However, transcription factors do not bind with the same specificity to long capture sequences as to short capture sequences. Indeed, increasing the length of the DNA sequence which contains a specific transcription factor binding site also proportionally and statistically increases the number of binding sites for the same, but mainly for other transcription factors. Hence, a spacer of 5 to 1 kb in length, as suggested by Heslot et al., or a DNA sequence of 18 to 250, as teached by Peterson et al., cannot be used to reach specific transcription factor detection.

12. To illustrate this purpose, the sequence of 46 bp given in example from Peterson et al., designed to bind NFkB (column 13, lines 10-12), was analyzed using the TFSEARCH engine (on WorldWideWeb at cbrc.jp/research/db/TFSEARCH.html; limitation to the vertebrate matrix). Two NFkB specific sites were identified (corresponding respectively to base pairs 10-19 and 31-40). However, binding sites for other factors were found to overlap these sites: NFkB site 1 is overlapped by sites for MZF1, GATA-3 and GATA-1, while NFkB site 2 is overlapped by sites for ADR1 and Ik-2.

13. Since Peterson et al. teach the possibility of use of sequences of 250 bp, the inventors tested for the feasibility of increasing the 46 base pairs used in Peterson et al. to a length of 250 bp naturally present in the gene containing the NFkB binding site. In a BLAST search performed on the 46 bp sequence of the exemple of Peterson et al., a list of sequences with high homology was obtained. The first sequence listed was the human sequence XM-941266-2 (bp 7357-7339), which has an identical to base pairs 24-42 of the test sequence, containing the NFkB binding site #2. We therefore considered a 250 bp fragment from this human sequence starting from position 7357 (7357-7108) to coveer the NFkB binding site and the adjacent sequence. Using the TFSEARCH analysis tool, 27 high score binding sites for transcription factors were identified in addition to the NFkB site (which now corresponds to bp 7341-7350), some overlapping or lying very close to this NFkB site. A second NFkB binding site was even identified (bp 7240-7249).

14. Considering such a sequence as a capture molecule would prevent the development of a specific NFkB assay, as transcription factors binding close to or within the NFkB binding would interfere with the assay. Such an assay would also not be reproducible, as different samples may contain different interfering transcription factors. Finally, quantification would not be possible, as the two NFkB sites from the example above have different sequences, and hence different affinities for the factor.

15. The present invention overcomes this problem by linking a short specific binding sequence to a spacer containing a double-stranded DNA nucleotide sequence of between 50 and 250 base pairs in length, and which is a nucleotide sequence not present in the cell containing the activated transcription factors to assay.

16. Exhibits 1-4 show experimental data obtained which exemplify the advantages of the claimed method.

17. Exhibit 1 shows that binding of activated transcription factors to a short specific binding sequences which are linked to a very short nucleotidic spacer of 6 bp is specific, but the obtained signals are in average very low. In the experiment of Exhibit 1, five activated transcription factors (NFkB, Elk-1, c-Myc, STAT1 and STAT3) were contacted with short double-stranded capture probes of 30 bp comprising the specific binding site for the target transcription factor and a spacer of 6 bp. The protocol provided in Example 3 of the present Application was used to

conduct the analysis, with several modifications. The probes were as in Example 1 of the application, except that the CMV spacer was replaced by a synthetic 5' aminated spacer. Spotting was performed directly on activated glass slides without streptavidin treatment, and the probes concentration was 2000 nM. The assay was conducted according to the protocol described in the TF Chip MAPK kit, with fluorescence detection (Eppendorf, Germany). Exhibit 1 compares the results obtained with the five transcription factors. As depicted in Exhibit 1, only NFkB is detected with good sensitivity, the other factors showing either a low signal (c-Myc, STAT1 and STAT3) or no signal at all (Elk-1). Results can be compared with the Exhibit 3 for longer spacers. The high variability in signal detection using 6 bp spacers is also incompatible with the simultaneous analysis of more than one factor. Thus, use of a short double-stranded DNA capture probe provides specificity but not sensitivity for the majority of the tested factors (4 out of 5).

18. Exhibit 2 shows that binding of an activated transcription factor (HNF3) to a short specific binding sequence which is linked to a long double-stranded DNA spacer of 100 bp may be specific or not depending on the sequence of the spacer. The short specific binding sequence of HNF3 is linked to the support via a spacer of 100 bp (spacer 1). Different spacer sequences of 100 bp (spacer 1 to 6) are also present on the array as such (not linked to HNF3 specific binding sequence). The protocol described in the TF Chip Stem Cell kit (Eppendorf, Germany) was followed in this experiment. As depicted in Exhibit 2, the HNF3 transcription factor binds specifically to its specific sequence linked to spacer 1, but also to spacer sequence 4 alone. Therefore, the selection of the spacer used in the assay is important to ensure sensitivity but also specificity. Such spacer must be designed so that it does not bind any transcription factor possibly interfering with the assay. Such spacer sequence is preferably a synthetic sequence not present in the cell assayed for the presence of activated transcription factor(s).

19. Exhibit 3 shows that binding of activated transcription factors to short specific binding sequences which are linked to a spacer comprising a double-stranded DNA sequence of between about 50 and about 250 base pairs, which is not present in the questioned cell, results in an assay which is highly specific and sensitive. The experiment was conducted as in Exhibit 1 but in addition to the spacer of 6 bp, synthetic spacers of 20, 50 and 100 bp were tested and the

resulting signals were quantified. As depicted in Exhibit 3, a high signal was obtained for the five tested transcription factors, when the specific binding sequence is linked to spacers of 50 and 100 bp. We also found that the signals measured with spacers below 50 bp may not increase linearly with the spacer size (see STAT3). This is important in the context of a micro-array because the binding conditions for all of the factors to be assayed on the array are uniform, while the optimal binding conditions for each factor are different. Enhancing the signal levels using spacers of the lengths recited in the claims offers the possibility to evaluate the binding of multiple factors under uniform conditions.

20. Exhibit 4 shows that even when a plurality of transcription factors are present in a sample, the use of a short specific binding sequence linked to a spacer comprising a double-stranded DNA sequence of between about 50 and about 250 base pairs in length, which is not present in the questioned cell, allows obtaining specific signals with high values (i.e. the method is sensitive and specific). Each capture probe of the micro-array contains double-stranded DNA comprising a specific binding site for the TF and a common spacer of 100 bp. Exhibit 4 shows the quantification of signals resulting from activated TFs binding to a micro-array (TF Chip MAPK, Eppendorf, Germany). Extracts were obtained from HeLa cells stimulated with PMA either for 10 min or for 1 hour, and the protocol was as described in the TF Chip MAPK kit instruction manual. In Exhibit 4, a signal increase was obtained for AP1 (c-Jun), MEF2 and p53 after a 1 hour PMA stimulation of HeLa cells compared to the 10 min stimulation condition. There was activation at both stimulation times for ATF2, cMyc and ELK1. NFATc1 and STAT1 were not activated at any stimulation time. The assay is quantitative and signal changes between different stimulation times (observed for AP1 (c-Jun), MEF2 and p53) are extremely valuable to understand the activation profile of a cell.

21. In conclusion, the method provided according the requirements of the present invention is both sensitive and specific even when a plurality of transcription factors is simultaneously quantified in a cell or cell lysate.

22. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: 10 December 2007 By: _____

Jose Remacle

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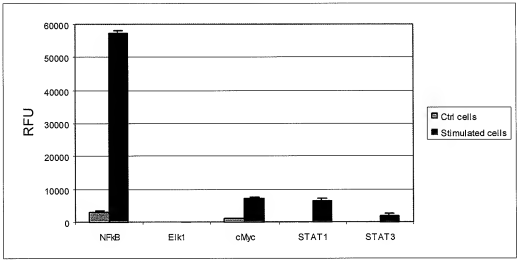

Exhibit 1: Quantification of signals obtained for the binding of five activated transcription factors (NFκB, Elk-1, c-Myc, STAT1, STAT3) to capture probes on a micro-array. Capture probes corresponding to each TF are made of double-stranded DNA and comprise a specific binding site and a common spacer of 6 bp. Signals for each TF were measured for control and stimulated cells in each factor's optimal assay conditions: NFκB: WI38 +/- interleukin-1; Elk-1: HeLa +/- phorbol 12-myristate 13-acetate (PMA); c-Myc: NIH3T3 +/- PMA; STAT1: COS7 +/- interferony; STAT3: HepG2 +/- interleukin-6. Signals were obtained with Cy3-labeled secondary antibodies and fluorescence scanning was performed using a ScanArray Express micro-array scanner from Packart BioScience and a laser power of 100. Scans were performed with a gain = 100, except for NFκB, where a gain = 80 was used. Y axis is in relative fluorescence units. X axis represents the transcription factor tested.

■: non-stimulated cells; ■: stimulated cells.

Ctrl+	SCL	HNF3
PPAR	MyoD	GATA
RUNX2	C/EBP	Oct4
STAT	p53	MEF2
Spacer1	Spacer2	Spacer3
Spacer4	Spacer5	Spacer6
HNF1	Ctrl-	Ctrl +

Exhibit 2: Fluorescent detection of the activated hepatocyte nuclear factor (HNF) 3 transcription factor through its binding to different capture probes on a micro-array.

Capture probes spotted on the array in triplicates:

- Short sequences specifically binding different transcription factors (26 bp) linked to spacer 1 (100 bp). The name of the transcription factor is given in blue.
- Spacers alone (Spacer1 to Spacer6; 100 bp) with different sequences (in red).

-Ctrl-: spotting buffer.

-Ctrl+: Cy3-labeled spacer 1.

The array was contacted with 30 µg of a nuclear extract from HepG2 cells. The primary antibody used was a goat anti-HNF3β polyclonal IgG (Santa Cruz Biotechnology). The assay was performed according to the procedure described in the instruction manual of the TF Chip Stem Cell kit (Eppendorf, Germany). Signals were obtained with Cy3-labeled secondary antibodies and fluorescence scanning was performed using a ScanArray Express micro-array scanner from Packart BioScience, a laser power of 100 and a gain of 80.

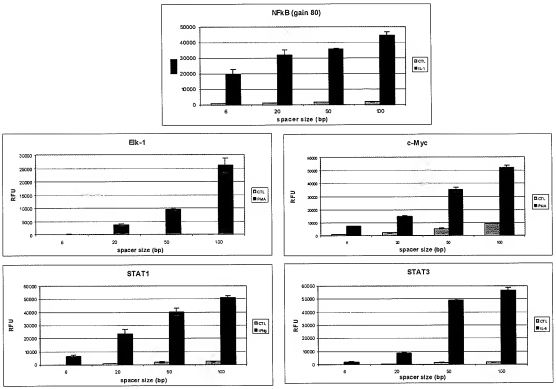

Exhibit 3: Quantification of signals obtained for the binding of five activated transcription factors (NFκB, Elk-1, c-Myc, STAT1, STAT3) on their respective capture probes on a micro-array as provided in Exhibit 1. Capture probes corresponding to each TF are made of double-stranded DNA and comprise a specific binding site and a common spacer of increasing length of 6, 20, 50 or 100 bp, which is a nucleotide sequence not present in the tested cells. Y axis is in relative fluorescence units. X axis represents the spacer size (bp).

■ : non-stimulated cells; ■ stimulated cells.

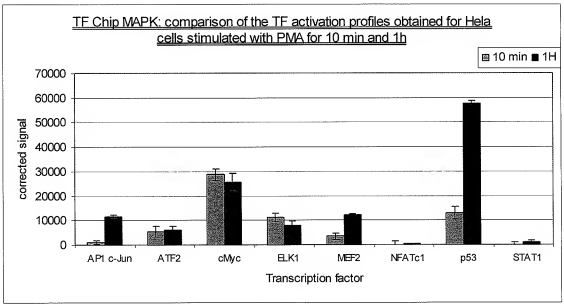

Exhibit 4: Quantification of signals obtained for the binding of activated transcription factors (TFs) in nuclear extracts of HeLa cells after PMA stimulation at two different times using the TF Chip MAPK kit (Eppendorf; Germany). Spots corresponding to each TF contain double-stranded DNA comprising a specific binding site for the TF and a common spacer of 100 bp. Signals for each TF were measured for 10 min stimulation with PMA (A) and for 1 hour stimulation with PMA (B). Signals were obtained with Cy3-labeled secondary antibodies and fluorescence scanning was performed using a ScanArray Express micro-array scanner from Packart BioScience and a laser power of 100. Scans were performed with a gain = 80. Y axis represents the fluorescence signals as generated by the TF Chip MAPK kit software. X axis represents the transcription factors that can be detected using the TF Chip MAPK kit.

▨ : 10 min stimulation; ■ : 1 hour stimulation.

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CREB: A STIMULUS-INDUCED TRANSCRIPTION FACTOR ACTIVATED BY A DIVERSE ARRAY OF EXTRACELLULAR SIGNALS

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Key Words CREB, CBP, PKA, CaMK, RSK, phosphorylation

■ **Abstract** Extracellular stimuli elicit changes in gene expression in target cells by activating intracellular protein kinase cascades that phosphorylate transcription factors within the nucleus. One of the best characterized stimulus-induced transcription factors, cyclic AMP response element (CRE)-binding protein (CREB), activates transcription of target genes in response to a diverse array of stimuli, including peptide hormones, growth factors, and neuronal activity, that activate a varicty of protein kinases including protcin kinase A (PKA), mitogen-activated protein kinascs (MAPKs), and Ca^{2+}/calmodulin-dependent protein kinases (CaMKs). These kinases all phosphorylate CREB at a particular residue, serine 133 (Ser133), and phosphorylation of Ser133 is required for CREB-mediated transcription. Despite this common feature, the mechanism by which CREB activates transcription varies depending on the stimulus. In some cases, signaling pathways target additional sites on CREB or proteins associated with CREB, permitting CREB to regulate distinct programs of gene expression under different conditions of stimulation. This review discusses the molecular mechanisms by which Ser133-phosphorylated CREB activates transcription, intracellular signaling pathways that lead to phosphorylation of CREB at Ser133, and features of each signaling pathway that impart specificity at thc levcl of CREB activation.

CONTENTS

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INTRODUCTION

Extracellular stimuli that trigger long-term phenotypic changes in cells, such as differentiation of a precursor cell or synaptic strengthening of a mature neuron, elicit changes in gene expression. These stimuli cause changes in gene expression by activating intracellular pathways that propagate the initial signal from the plasma membrane to the nucleus. These pathways are comprised of protein kinase cascades that culminate in the phosphorylation and activation of critical transcription factors. Cyclic AMP (cAMP)-responsive element (CREB)—bind-

ing protein is perhaps the best characterized stimulus-induced transcription factor. CREB was originally identified as a target of the cAMP signaling pathway, but studies on activation of immediate-early genes (IEGs) (1) revealed that CREB is a target of other signaling pathways activated by a diverse array of stimuli. All signaling pathways that activate CREB lead to phosphorylation of a particular residue, serine 133 (Ser133). Phosphorylation of Ser133 is required for CREB-induced gene transcription; however, CREB activity and specificity can be further modulated by phosphorylation of additional sites on CREB or of proteins associated with CREB.

The structure of CREB and its relatives and corresponding genes have been extensively reviewed elsewhere (2, 3). This review focuses on the molecular mechanisms governing stimulus-induced activation of CREB and CREB family members. CREB functions as a stimulus-induced transcriptional activator in organisms ranging from *Aplysia* to *Drosophila* to man (4). Studies on the biological functions of CREB have shown that CREB is critical for a variety of cellular processes, including proliferation, differentiation, and adaptive responses. In mice, CREB is required during development for generation of a normal repertoire of T-cell lineages (5), and absence of CREB leads to dwarfism and cardiac myopathy in the adult (5a, 6). CREB family members are believed to be important for learning and memory (7) and contribute to neuronal adaptation to drugs of abuse (8). CREB activity is also important for hormonal control of metabolic processes, including regulation of gluconeogenesis by the hormones glucagon and insulin (9).

This review discusses findings on stimulus-induced CREB activation from experiments conducted primarily in mammalian systems. However, given the conservation across species within regions of CREB involved in stimulus-induced transcription (10), it is likely that mechanisms revealed for mammalian CREB will apply to CREBs from other species.

THE CREB FAMILY

The Discovery of CREB

Identification and characterization of CREB stemmed from studies of cAMP regulation of peptide hormone biosynthesis, in particular from somatostatin expression in response to other hormonal stimuli. In mammals, circulating hormones such as glucagon and epinephrine cause an increase in the intracellular level of cAMP in target cells (11). The cAMP can, in turn, stimulate release of somatostatin from the appropriate cell type (12, 13). These circulating hormones also stimulate transcription of the genes for somatostatin and other neuropeptides (14, 15). Initial efforts to understand the molecular basis of cAMP-dependent induction of somatostatin gene transcription focused on deletion analysis of the promoter. This analysis identified an 8-bp cAMP-responsive element (CRE), 5′-TGACGTCA-3′, that is critical for cAMP induction of a reporter gene fused

to the somatostatin promoter (referred to as a somatostatin reporter gene) (15). The CRE was subsequently shown to bind CREB, present in nuclear extracts, and CREB was purified from nuclear extracts of the pheochromocytoma cell line PC12 by using CRE affinity chromotography and shown to be a 43-kDa phosphoprotein (16). A human CREB cDNA was cloned by screening a human cDNA expression library with a double-stranded ^{32}P-labeled CRE (17). Independently, sequence analysis of purified rat CREB led to the cloning of rat CREB cDNA (18). The human clone, termed CREBΔ, encodes a 327-residue protein, whereas the rat clone encodes a 341-residue protein termed CREBα (all numbering of residues hereafter refers to position in CREBα). The two forms differ only by the presence of a 14-residue insert, termed the α-peptide, in the longer form (19). Both forms of CREB are present in human, rat, and mouse tissue, and both are ubiquitously expressed in somatic cells (20–22). The mouse and human CREB genes are each composed of 11 exons, with alternative splicing of one exon (the exon encoding the α-peptide) generating CREBα and CREBΔ (19, 23, 24). Another recently identified isoform, CREBβ, is generated by alternative splicing of several 5′ exons, creating a CREB protein lacking the first 40 residues of both CREBα and CREBΔ (25) (Figure 1). Like CREBα and CREBΔ, CREBβ is uniformly expressed in all tissues. Several other far less abundant forms of CREB are also generated via alternative splicing, and some of these transcripts are expressed at particularly high levels in the testis (22, 24, 26). Although CREBα, CREBΔ, and CREBββ can all activate CRE-dependent transcription in response to elevated levels of cAMP, the biological functions of the other CREB isoforms have not been well characterized. However, available data suggest that certain alternatively spliced exons present in these rarer CREB isoforms generate inhibitory forms of CREB that might be important for regulating expression and activity of the active CREB isoforms in germinal cells (24). Screening of cDNA expression libraries has also identified a novel, thymus-specific CREB isoform in which alternative splicing of one exon results in the deletion of the α-peptide as well as the deletion of an additional 62 residues (183–244) (27).

ATF-1 and CREM

Subsequent to characterization of CREB, two other highly related gene products were characterized: activating transcription factor 1 (ATF-1) and cAMP response element modulator (CREM). ATF-1 was identified originally as one of several factors that bind to CRE-like elements present in adenovirus promoters (28–31). Subsequent screening of a HeLa cDNA library with CRE probes identified a large family of ATFs of which one, ATF-1, is 65% identical to CREB in primary structure (32). Examination of ATF-1 mRNA in different cell lines derived from a variety of tissue types suggests that ATF-1 is widely expressed (33). Screening of a mouse pituitary cDNA library with a probe corresponding to the DNA-binding and dimerization domain of CREB led to isolation of a series of novel cDNAs all derived from a single gene. This gene, CREM (34), has extensive sequence identity with CREB (22, 35). One particularly interesting characteristic of CREM is

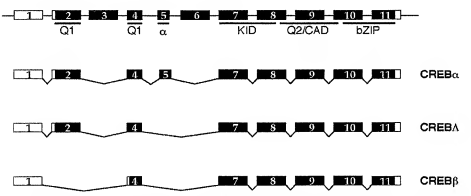


Figure 1 Principal cAMP response element-binding protein (CREB) isoforms. The genomic structure of the mouse CREB gene is shown, with the three most common alternatively spliced isoforms represented below. (*Solid boxes*) These are coding exons. (*Open boxes*) These are noncoding exons. (*Solid lines*) These indicate splicing events. The domains of the CREB protein (Q1, Q2, α, KID, Q2/CAD, and bZIP) corresponding to particular exons are shown in the schematic on *top*. Adapted from Blendy et al (25). Printed with permission of Oxford University Press.

that by alternative splicing, the same gene generates either activator or repressor forms of CREM. The α, β, and γ isoforms of CREM all bind to CREs, but function as inhibitors of CREB and cAMP-mediated transcription (34). However, splicing of two additional exons generates a CREM isoform, CREMτ, which is the most similar to CREB with respect to amino acid sequence, that functions as an activator of CRE-mediated transcription (35–37). Two particularly short forms of CREM are generated through use of either an internal translation initiation site or an internal intronic promoter (38–40). The resulting products are essentially composed of a DNA-binding and dimerization domain and function as CRE-binding repressors. Unlike CREB, the various isoforms of CREM are not expressed uniformly across different tissues (35). CREMτ mRNA is particularly enriched in testis, with some expression in brain (36), whereas expression of the truncated CREM isoforms appears to be confined to tissues of the neuroendocrine axis (38, 39).

The bZIP Superfamily

CREB binds to its DNA target sequence as a dimer (41). Dimerization occurs through a conserved structural motif at the C-terminus of the protein (Figure 2) formed by a heptad repeat of leucine residues, referred to as the leucine zipper (42, 43). DNA binding is mediated by a basic domain, a lysine- and arginine-rich stretch of amino acids just amino-terminal to the leucine zipper (43). Presence of both the basic and leucine zipper (bZIP) domains places CREB within a larger family of bZIP transcription factors, including mammalian c-Fos, c-Jun, c-Myc, and C/EBP, as well as yeast Gcn4 (44, 45).

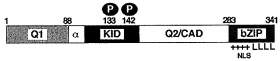


Figure 2 Domains of CREB. Shown are the principal domains of the CREB protein, along with the phosphorylation sites at Ser133 and Ser142. *Numbers on top* indicate the position of amino acid residues, (+) marks refer to the positively charged basic domain, and L refers to the leucine zipper domain. The position of the nuclear localization signal (NLS) is also indicated.

Among the bZIP factors, CREB, ATF-1, and CREM constitute a subgroup of proteins that share a high degree of identity within the bZIP region. CREB and ATF-1 are 91% identical in the bZIP domain (32, 33). The CREM gene contains two bZIP domains; alternative splicing determines which bZIP is present in a particular CREM isoform. One of the CREM bZIP domains shares 95% identity with CREB, whereas the other bZIP region exhibits only 75% identity (34). Like CREB, ATF-1 and CREM are able to bind to CREs as homodimers (32, 34, 35). However, the presence of highly related dimerization domains suggested that different members of the CREB/ATF-1/CREM subgroup might be able to form heterodimers. CREB/CREM heterodimers have been detected on cognate CREs in vitro (34, 35, 46). ATF-1/CREB heterodimers have been detected in a variety of cell lines, although the amount of heterodimer relative to homodimers varies depending on the cell type (31, 47–49). Compared with ATF-1/ATF-1 homodimers, ATF-1/CREB heterodimers have a longer half-life when bound to the CRE (both approximately several minutes), whereas CREB/CREB homodimers have the longest half-life (10–20 min). This duration of a CREB-dependent transcriptional event may be determined, at least in part, by the nature of the dimer bound to the target promoter (48).

While CREB, CREM, and ATF-1 are able to form heterodimers with each other and may heterodimerize with certain other bZIP factors, such as NF-IL6 (see below), CREB family members do not heterodimerize with many other bZIP classes. Thus, although c-Jun and c-Fos can heterodimerize efficiently (50) and although other ATFs (including ATFs 2, 3, and 4) can heterodimerize with each other as well as with Fos and Jun family members (32, 51), neither CREB nor ATF-1 appear to do so (42, 52). These data support the idea that the CREB/ATF-1/CREM family represents a distinct group, with each factor having the potential to regulate the activity of the others. Which of the six possible CREB, CREM, and ATF-1 combinations actually form, where and when they form, and the nature of their activity are unexplored issues.

PHOSPHORYLATION-DEPENDENT ACTIVATION OF CREB

Stimulus-induced activation of CREB is mediated by phosphorylation. Exposure of cells to forskolin (53), an activator of adenylyl cyclase, leads to CREB phosphorylation at a specific residue, serine 133 (Ser133); this site is phosphorylated in vitro by cAMP-dependent protein kinase A (PKA) (16, 18, 54). Phosphorylation of Ser133 is required for signal-induced transcription in vivo, as mutation of Ser133 to a nonphosphorylatable residue (alanine) abolishes transcriptional response to elevated cAMP (54).

How does phosphorylation of a transcription factor stimulate its ability to activate transcription (55)? Phosphorylation at Ser133 could affect the stability of CREB, so that it is less labile when phosphorylated. Because CREB functions in the nucleus, phosphorylation at Ser133 might induce translocation of cytoplasmic CREB to the nucleus. Within the nucleus, phosphorylation at Ser133 might affect the ability of CREB to dimerize with different bZIP partners, or phosphorylation at Ser133 might promote CREB binding to the CRE. Finally, phosphorylation at Ser133 might lead to transcriptional activation by promoting interaction with components of the basal transcription machinery, such as TFIID and RNA polymerase II (Pol II), or other factors. There is evidence for and against each of these regulatory mechanisms and these findings suggest which particular mechanisms are most likely to contribute to phos-phorylation-dependent activation of CREB.

Regulation of Protein Stability

Phosphorylation has been shown to affect stability of certain transcription factors, such as c-Jun, by decreasing their ubiquitin-dependent degradation (56). However, CREB activates stimulus-dependent transcription within 30 min, while immunoblot analysis shows that the total level of CREB is unchanged even after several hours (57–59). Therefore, it is unlikely that any effect on CREB turnover alone can account for the rapid kinetics of CREB-dependent transcriptional induction.

Regulation of Subcellular Localization

The nuclear localization signal of CREB maps to a basic nine- residue stretch within the DNA-binding domain (60). For some transcription factors, phosphorylation regulates nuclear localization (61). For example, in yeast phosphorylation of the transcription factor, PHO4 blocks its ability to interact with nuclear import proteins (61a), resulting in its cytoplasmic retention and the inhibition of target gene transcription (61b). Immunostaining of cells and immunoblotting of subcellular fractions show that CREB is almost exclusively nuclear in both unstimulated and stimulated cells (60, 62, 63). However, the existence of rare alternatively-spliced variants of CREB that lack the bZIP domain and are not

localized to the nucleus raises the possibility that they may have some role in the cytoplasm (24, 26). One study has shown that CREB can be localized in the dendrites of neurons, well away from the nucleus (64), raising the possibility that stimulation of the dendrites (by neurotransmitter release from the presynaptic neuron) could trigger CREB translocation to the nucleus.

Regulation of Dimerization

Although one report has suggested that protein kinase C (PKC) phosphorylation of CREB regulates its dimerization (41), there remains no compelling evidence that homodimerization of CREB is affected by phosphorylation. However, indirect evidence suggests that different stimuli may lead to association of CREB with other bZIP partners. For example, when a plasmid encoding a Gal4-CREB fusion (containing a fusion of full-length CREB protein with the dimerization and DNA-binding domain of the yeast transcription factor Gal4) is transfected into cells along with a Gal4-dependent reporter gene (a plasmid containing Gal4-binding sites in its promoter), the Gal4 fusion protein activates Gal4 transcription effectively in response to a calcium (Ca^{2+}) stimulus, but only when the CREB leucine zipper is intact (57, 65). This result suggests that although Gal4-CREB forms a homodimer via the dimerization domain provided by Gal4, the leucine zipper of the CREB fusion may need to associate with an endogenous leucine zipper-containing protein to activate transcription in response to a Ca^{2+} stimulus. The endogenous leucine zipper protein could be ATF-1 or CREM, both of which can heterodimerize with CREB, as mentioned above. However, stimulus-induced heterodimerization of CREB with either of these two factors has not been reported. It is conceivable that CREB heterodimerizes with a bZIP factor distinct from either the Fos/Jun or the CREB families. This factor, NF-IL6 (also referred to as C/EBPβ), may be a CREB partner whose interaction with CREB is induced by extracellular stimuli. In response to elevated levels of cytoplasmic Ca^{2+}, NF-IL6 becomes phosphorylated at a serine within its leucine zipper (66), which may promote NFIL6/CREB heterodimerization because CREB/NF-IL6 heterodimers have been observed after other stimuli (67).

Regulation of DNA Binding

The K_d of the CREB-CRE interaction has been reported to range from 1 to 180 nM. One parameter in particular that affects the affinity of CREB for its cognate DNA element is the exact sequence of the CREB-binding site. In one study using an electrophoretic mobility shift assay, CREB bound to a symmetric CRE (from the somatostatin gene) with >10-fold–higher affinity than to an asymmetric CRE (from the tyrosine aminotransferase gene) (68). CREB Ser133 phosphorylation did not change the affinity of CREB for the somatostatin CRE, but greatly increased the affinity of CREB for the tyrosine aminotransferase *(TAT)* CRE. Genomic foot-printing has also revealed that elevated levels of

cAMP increase occupancy of the *TAT* CRE in vivo (69). Another study, using a fluorescent electrophoretic-mobility shift assay, found that CREB binds with 10-fold greater affinity to a symmetric CRE than to an asymmetric CRE, but phosphorylation of Ser133 doubled the binding affinity to both targets (70). A subsequent study, using fluorescence anisotropy, suggests that phosphorylation at Ser133 has no effect on the affinity of CREB for the *TAT* CRE in vitro (71). Several other studies have also failed to detect phosphorylation-induced changes in the CREB/DNA interaction, with respect to both the somatostatin CRE and other CREB-binding elements, such as the Tax-responsive element (63, 72). Hydrodynamic analysis shows that CREB is primarily monomeric in solution, that CREB dimer formation is strongly promoted by the presence of CRE DNA, and that this dimerization is independent of phosphorylation (73, 74). It is unclear why different laboratories have reached such disparate conclusions. The concentration of CREB in the nucleus of PC12 cells has been calculated to be ~400 nM (63). At this concentration the high-affinity CREs would be predicted to be nearly saturated and thus even if Ser133 phosphorylation increases DNA-binding affinity, little effect would be expected. In contrast, weaker CREB-binding sites might have much less CREB bound, and theoretically Ser133 phosphorylation might stimulate transcription by increasing the frequency of occupancy (68).

Regulation of Transcriptional Activity

As discussed above, CREB can activate transcription in response to a stimulus when recruited to a promoter through a heterologous DNA-binding domain (65). Thus, CREB activity is regulated primarily by processes other than DNA-binding. Ser133 is located within a 60-residue region of CREB, called the kinase-inducible domain (KID) (Figure 2). The KID (residues 100–160) element encompasses multiple potential phosphorylation sites for various protein kinases (75). The KID is both necessary and sufficient for signal-induced activation of CREB: A Gal4-KID fusion that lacks all other domains of CREB still activates transcription in response to various stimuli (76, 77) and deletion of specific regions within the KID greatly diminishes stimulus-induced activation (75, 77).

How does phosphorylation of the KID result in transcriptional activation? To search for factors that associate with CREB in a phosphorylation-dependent manner, a human thyroid cDNA expression library was screened with ^{32}P-labeled CREB and a protein was isolated that specifically bound to Ser133-phosphorylated CREB (78). This factor, CREB-binding protein (CBP), is a 265-kDa nuclear protein that associates with phosphorylated CREB through a region at the N terminus of CBP known as the KID interaction (KIX) domain (78). The core KIX domain is a 94-residue sequence (positions 586–679) (79). The same residues of KID most critical for transcriptional activation by CREB (positions 140 to 160) are also required for interaction of CREB with the KIX domain of CBP, suggesting that CBP binding is important for CREB activity.

Other data support the idea that CBP is critical for stimulus-induced activation of CREB: (*a*) coexpression of CBP increases stimulus-induced CREB transcription of a CRE reporter gene, an effect that is lost when Ser133 is mutated to an alanine (80); (*b*) microinjection of cells with neutralizing anti-CBP antibodies inhibits cAMP-induced activation of a CRE reporter gene (81); and (*c*) microinjection of a KIX peptide into cells inhibits stimulus-induced activation of a CRE reporter gene, presumably because the KIX peptide competes with CBP for interaction with CREB (79). The KID/KIX interaction may not be sufficient for CREB-mediated transcription because it is possible to mutate a particular residue within the CREB KID and inhibit transcriptional activation without affecting CBP binding (82).

Structure of Ser133-phosphorylated CREB KID complexed to the CBP KIX domain has been solved using NMR spectroscopy and suggests how phosphorylation promotes association (83). In the absence of phosphorylation or of binding to the CBP KIX domain, the KID of CREB is not highly ordered. However, when phosphorylated at Ser133 and bound to the KIX domain of CBP, the KID assumes a structure consisting of two α-helices that kink close to the phosphorylation site at Ser133 (Figure 3). The helix C terminal to the kink is bound tightly within a hydrophobic pocket created by a palisade of three α-helices of the KIX domain. This hydrophobic interaction presumably contributes significantly to the KID/KIX domain interaction because functional data show that the corresponding segment of CREB (residues 140–144) is critical for transcriptional activation in vivo (75, 84).

CBP shares extensive sequence similarity throughout its length with another protein, termed p300, which was initially characterized as an adenovirus E1A-associated protein (85–87). Although CBP and p300 have very similar actions in mediating CREB function, the use of ribozymes to inhibit production of p300 or CBP in vivo has revealed functional distinctions between these two co-activators at the level of target gene expression (88). Moreover, cAMP-mediated activation of CRE-dependent gene expression is not reduced in fibroblasts derived from p300-deficient mice, suggesting that association of p300 with Ser133-phosphorylated CREB is not required for CREB activity in every cell type (89).

CBP ACTIVATION OF CREB

Transcriptional Adaptor

Evidence suggests that CBP serves as a molecular bridge that allows upstream transcription factors, such as CREB, to recruit and stabilize the RNA polymerase II (Pol II) transcription complex at the TATA box. In this regard, CBP shares regions of sequence similarity with other proteins known to function as transcriptional adaptors. In particular, CBP contains a cysteine-rich, zinc finger

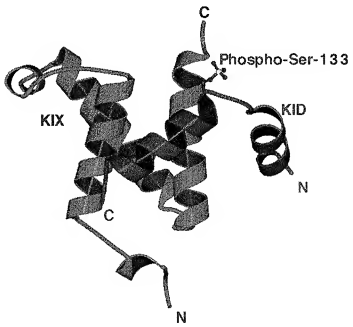


Figure 3 Ribbon diagram of the interaction between the phosphorylated KID domain of CREB and the KIX domain of CBP. The NMR-deduced structure of the KIX domain of CBP (*turquoise*) bound to the Ser133–phosphorylated KID domain of CREB (*pink*). The KIX domain comprises residues 586–666 of mouse CBP, whereas the structured region of the KID domain comprises residues 119–146 of CREB. The phosphoserine residue is shown in ball-and-stick representation. The KID domain undergoes a folding transition on binding to KIX, forming two α-helices in the process. The C-terminal helix of KID binds to a hydrophobic surface formed by helices α-1 and α-3 of KIX. Diagram courtesy of P Wright and I Radhakrishnan.

domain homologous to a region found in the yeast Ada2 protein, an adaptor that physically links yeast transcriptional activators to the general transcriptional machinery (90, 91). CBP also has a bromodomain, a region found in other transcriptional adaptors, such as the TATA box-binding protein (TBP)-associated factor, $TAF_{II}250$, and the *Drosophila* and mammalian homologs of yeast Swi2/Snf2 protein (92, 93). The idea that CBP acts as a transcriptional adaptor linking Ser133-phosphorylated CREB to the basal transcription machinery is supported by biochemical evidence. CBP cofractionates with Pol II during ion-exchange and gel-filtration chromatography of HeLa cell nuclear extracts, and both CBP and Pol II co-immunoprecipitate with Ser133–phosphorylated CREB (94). Pol II recruitment to the CREB/CBP complex requires that the KID be phosphorylated, and in vitro experiments suggest that

the Ser133–phosphorylated KID is both necessary and sufficient for Pol II recruitment in vitro (95). In addition to the KIX domain, a specific C-terminal region of CBP (the C/H3 domain) is required for interaction with Pol II. Although CBP can associate with Pol II directly (96), in vivo interaction may be indirect. Screening of a cDNA expression library with the C/H3 domain of CBP identified a factor, RNA helicase A (RHA), that binds to the C/H3 domain of CBP and appears to mediate its interaction with Pol II (97). All three factors—CBP, RHA, and Pol II—were found to coimmunoprecipitate with each other from whole-cell extracts. Moreover, coexpression of both RHA and CBP greatly enhances the ability of PKA-stimulated CREB to activate transcription. Thus, phosphorylated CREB uses two bridging proteins, CBP and RHA, to stabilize its interaction with Pol II (Figure 4).

Effects on Chromatin

In addition to recruiting Pol II, CBP also contributes to CREB-mediated transcription by affecting chromatin structure. CBP possesses an intrinsic histone

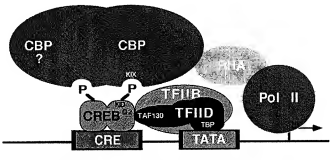


Figure 4 Multiple domains of CREB contribute to transcriptional activation. Different domains of CREB bind distinct coactivators and basal transcription factors to activate transcription. Shown is a CREB dimer bound to its cognate CaRE/CRE element on the promoter of a CREB target gene. Downstream of the CaRE/CRE is the TATA box, which binds the multiprotein TFIID basal transcription factor (via the TBP protein). Another factor within TFIID, TAF130, binds to the Q2 domain of CREB. The Q2 domain of CREB has also been shown to interact with TFIIB, which is a part of the basal transcription machinery as well. A distinct domain of CREB, the KID, contributes to signal-induced transcriptional activation. When phosphorylated at Ser133, the KID of CREB can bind to the KIX domain of the CBP. It is presently unclear whether CBP associates with Ser133–phosphorylated CREB as a dimer. CBP associates indirectly with Pol II via the RNA helicase A (RHA) protein. Therefore, recruitment of CBP to Ser133–phosphorylated CREB results in recruitment and stabilization of Pol II on the promoter of CREB target genes, whereas the Q2 domain interacts with other elements of the basal transcription machinery that are required for transcription, such as TFIID and TFIIB.

acetyltransferase (HAT) activity and associates with another HAT-containing factor termed p/CAF (98–100). By catalyzing acetylation of lysine residues in the N-termini of histones, CBP and p/CAF alter chromatin structure in a fashion believed to make the DNA template more accessible to the transcriptional machinery (101). The mechanism through which this occurs has not yet been clearly elucidated. Microinjection studies using function-blocking antibodies followed by complementation with expression vectors that direct the overexpression of the requisite protein have suggested that the intrinsic HAT activity of CBP, but not p/CAF, is required for CRE-dependent transcription in vivo (102). Thorough understanding of the requirement for chromatin modifying factors to achieve CREB-dependent transcription awaits more precise perturbation of the HAT activities of CBP and p/CAF, so that the effects of these disruptions on chromosomal gene expression (as opposed to extrachromosomal reporter genes) can be examined.

Relevance to Normal Development and Disease

CBP is important for both development and proliferation. In humans, loss-of-function mutations in one allele of CBP result in Rubinstein-Taybi syndrome, a syndrome characterized by mental retardation, broad thumbs, and increased risk of the proliferative disorder known as keloids (103). Certain skeletal anomalies seen in Rubinstein-Taybi syndrome are also observed in mice heterozygous for CBP (CBP+/-) (104). Since CBP associates with a large number of transcription factors in addition to the CREB family (105), it is difficult to conclude at the present time if the effects of mutating CBP on development reflect a perturbation of CREB-mediated events and/or disruption of the expression of other genes.

FUNCTIONAL CONTRIBUTIONS OF OTHER DOMAINS OF CREB

The KID is clearly able to function as an independent module in mediating a stimulus-induced transcriptional response (76, 77). However, the level of the transcriptional response is modest unless other domains of CREB are also present (75). In particular, a different region of CREB has been identified as important for CREB activity under circumstances where CREB functions as a regulator of transcription in the absence of a stimulus (76, 77). This glutamine-rich domain (referred to as the Q2 or CAD) encompasses residues 165–252 of CREB (see Figure 2) and is necessary for basal, but not signal-induced, activation (106). The Q2/CAD domain physically interacts with components of the general Pol II transcription machinery, including TFIIB and the $TAF_{II}130$ subunit of TFIID (107, 108). $TAF_{II}130$ has been shown to be required for CREB-mediated transcription in vitro (95). Interestingly, the transcription factor, Sp1, associates with the *Drosophila* homolog of $TAF_{II}130$, $dTAF_{II}110$, through a hydrophobic patch in Sp1 that is very similar to a sequence within the Q2/CAD of CREB (109).

This similarity suggests that domains related to Q2/CAD may play a more general role in the recruitment of TFIID during formation of the Pol II transcription complex. A glutamine-rich region (Q1) within the N terminus of CREB also contributes to CREB's transcriptional activation potential, although the magnitude of this contribution is modest (75, 77).

ATF-1 differs from CREB throughout most of its N terminus, and, therefore, ATF-1 contains no Q1-like domain (33). However, ATF-1 shares extensive homology to CREB throughout most of its C terminus, including the Q2/CAD region. The active CREMτ isoform is highly homologous to almost the entire CREB protein, including both Q1 and the Q2/CAD (35, 36). By contrast, the repressor isoforms of CREM have no similarity to either the Q1 or Q2 regions of CREB, further supporting the conclusion that Q1 and Q2 are important for transcriptional activation.

The findings discussed above indicate that the KID and factors such as CBP that associate with the KID synergize with other CREB domains to mediate robust transcriptional activation. What is particularly interesting is the modular nature of this synergy: The two modules (KID and Q2/CAD) need not be part of the same molecule—they are able to cooperate in *trans*. This synergy was shown by coexpression of a Gal4-KID fusion protein with another Gal4 fusion protein containing the Q2/CAD (76). In fact, the KID can cooperate with constitutive activator domains from unrelated transcription factors, such as yeast Gal4 and Gcn4. These findings confirm that CREB uses multiple mechanisms to interact with and stabilize the Pol II transcriptional machinery. While the KID recruits Pol II activity via its interaction with CBP, additional basal factors recruited through other domains of CREB are also needed to stabilize TFIID and induce optimal transcriptional response (Figure 4).

TOOLS FOR EXAMINING CREB ACTIVATION AND FUNCTION

Phospho-Specific Antibodies

The ability to monitor easily phosphorylation of CREB at Ser133 in vitro and in vivo has proved to be important in identifying the signaling pathways that trigger CREB phosphorylation and activation, and has been provided by the development of antibodies specific for Ser133–phosphorylated CREB (phospho-CREB) (62, 63). Anti-phospho-CREB antibodies have numerous advantages over phosphopeptide analysis, the alternative method for determining the in vivo phosphorylation state of proteins. In addition to avoiding the use of ^{32}P, which may be a cellular stress due to radiation-induced damage, use of anti-phospho-CREB antibodies allows for convenient determination of the phosphorylation status of Ser133 under different circumstances. Whereas phosphopeptide analysis requires cell lysis, phospho CREB antibodies can be used in immunocytochemistry assays

for analysis of CREB phosphorylation at the single-cell level in both cultured cells and tissue sections from animals (62).

Dominant-Negative Mutants

The use of dominant interfering mutants of CREB has facilitated investigation of the involvement of CREB in gene induction in response to any given intracellular signaling pathway. To date, three dominant interfering CREB mutants have been used: non-phosphorylatable S133A, referred to as M1 CREB (54); a K287L, within the DNA-binding domain of CREBΔ, referred to as K-CREB (110); and, a mutant in which basic residues within the bZIP domain have been mutated to acidic residues, referred to as A-CREB (111). M1, although unphosphorylatable (54), can still bind to the CRE. Thus, M1 inhibits CREB action by occupying the CRE and preventing access by CREB and other CRE-binding factors. K-CREB is unable to bind to the CRE, but is able to dimerize with itself and endogenous wild-type CREB. Thus, K-CREB blocks gene activation by titrating endogenous CREB family members and preventing their interaction with the CRE. A-CREB also functions by heterodimerizing with endogenous CREB family members and preventing their interaction with the CRE (111). Its acidic patch is thought to mimic DNA and bind to the basic region of a wild-type CREB partner, thereby preventing its binding to the CRE. Heterodimers of A-CREB and wild-type CREB are orders of magnitude more stable than homodimers of either CREB or A-CREB, thereby ensuring that, within a cell, A-CREB and CREB will interact preferentially.

Although each of these CREB mutants can inhibit the function of endogenous CREB, their mechanisms of action may produce undesired side effects. For example, overexpression of M1 may result in its binding to sequences that are not normally occupied by endogenous CREB, so that not all genes whose expression is inhibited will be bona fide CREB targets. K-CREB and A-CREB have the advantage that they function off the DNA and therefore will most likely not occlude binding of transcription factors near the CRE. On the other hand, because CREB, ATF-1, and CREM share almost identical bZIP domains, K-CREB and A-CREB and even M1, when overexpressed, would be expected to interfere with both ATF-1– and CREMτ-dependent transcription. The use of mutants that act by different mechanisms eliminates some of these problems and permits assessment of the role of CREB in particular cellular responses.

SIGNALING AND CREB

Many agents, including neurotransmitters and hormones, act to increase the intracellular level of cAMP. Typically, such a ligand binds to its cognate receptor, most often of the seven-transmembrane domain class of receptors (111a). Ligand binding to the receptor leads to the activation of a coupled heterotrimeric G-protein, whose activated subunits stimulate one or more of the adenylyl cyclase

isoforms, the enzymes catalyzing cAMP production (112). Production of cAMP is counteracted by the action of phosphodiesterases, the enzymes that cleave cAMP. In most cells, the primary target of cAMP is the cAMP-dependent protein kinase (PKA). In the absence of cAMP, PKA consists of an inactive heterotetramer of two catalytic subunits bound to two regulatory subunits (113). Upon binding cAMP, the regulatory subunits dissociate and release the catalytic subunits, which are now free to phosphorylate target proteins. The released catalytic subunits may also translocate to the nucleus (63), and the best characterized nuclear substrates of PKA are CREB family members (Figure 5). Pharmacological studies with PKA inhibitors implicated PKA as the mediator of cAMP's effects on gene expression. In particular, PKA was shown to be required for forskolin induction of transcription of somatostatin promoter-driven reporter genes (15). PKA activation of the somatostatin promoter required an intact CRE, thus implicating CREB as a mediator of the effects of PKA. Subsequent studies have identified a wide range of genes induced by elevated levels of cAMP through a PKA/CRE-dependent mechanism (10).

Kinetics of CREB Activation and Inactivation

The kinetics of PKA translocation to the nucleus, CREB Ser133 phosphorylation, and somatostatin transcriptional activation support the hypothesis that forskolin induces somatostatin transcription via PKA phosphorylation of CREB. In PC12 cells exposed to forskolin, the PKA catalytic subunits accumulate in the nucleus within 15–30 min (63). This timing closely parallels the observed appearance of somatostatin transcription (114). Phosphorylation of CREB at Ser133 also proceeds with similar kinetics. The purified catalytic subunit of PKA phosphorylates CREB at Ser133 in vitro with $K_m = 4.7\,\mu$ M and $V_{max} = 0.82\,\mu$mol min^{-1} mg^{-1} (115). CREB phosphorylated in vitro by PKA can stimulate CRE gene expression when microinjected into fibroblasts (116). Taken together with the finding that mutation of CREB Ser133 to alanine blocks PKA stimulation of CREB-dependent transcription, it demonstrates that PKA phosphorylation is required for CREB activity. However, these experiments do not rule out that additional modifications of CREB might also be necessary for the full cAMP response.

Most cAMP-regulated genes are induced transiently (114, 117). For example, somatostatin transcription peaks 30 min after forskolin treatment and declines to baseline levels within 4 hours. Dephosphorylation of Ser133 parallels the shutoff of somatostatin transcription (114). Several serine/threonine phosphatases could mediate dephosphorylation of Ser133 (118). Two okadaic-acid–sensitive phosphatases, protein phosphatase-1 (PP-1) in PC12 cells (114) and protein phosphatase PP-2A in HepG2 cells, have been reported to be CREB phosphatases (114, 119).

Other mechanisms contribute to regulation of CREB phosphorylation. For a prolonged period, starting several hours after stimulation and lasting for up to several days, there is a refractory period during which cAMP is unable to reactivate transcription of target genes (120, 121). This period corresponds to down-

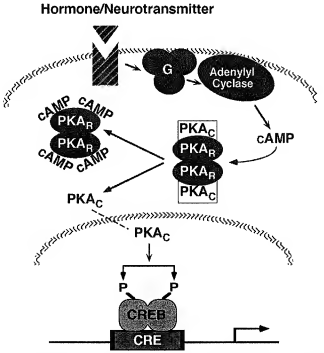


Figure 5 Activation of CREB by the cAMP/PKA signaling pathway. Hormones and neurotransmitters can raise intracellular cAMP levels by binding to receptors that activate heterotrimeric G-proteins (G). G-proteins then directly activate adenylyl cyclase, which catalyzes the production of cAMP. The cAMP leads to the activation of PKA. In the absence of cAMP, PKA consists of a tetramer of two catalytic subunits of PKA (PKA_C) bound by a dimer of two regulatory subunits (PKA_R). In this form, the PKA_C subunits are inactive. When intracellular cAMP levels are elevated, four molecules of cAMP bind to the dimer of PKA_R, releasing active PKA_C. Active PKA_C then translocates to the nucleus (*dashed line*) where it phosphorylates CREB at Ser133.

regulation of PKA catalytic subunit expression and can be reversed by exogenously introduced PKA (121). Other studies have shown that the duration of the refractory period correlates with the length of pretreatment with the initial stimulating agent and have suggested that the refractory period results, in part, from induction of repressors, including an isoform of CREM, termed ICER (inducible cAMP early repressor) (122).

The duration of Ser133 phosphorylation determines the efficacy with which CREB induces gene expression. In a subtype of striatal neurons, stimulation with a cAMP-activating neurotransmitter, dopamine, evokes Ser133 phosphorylation

that is maintained even 30 min post-stimulation (123). In other subtypes of striatal neurons, however, cAMP signals induce more transient phosphorylation of CREB such that Ser133 is already dephosphorylated within 15 min of the initial stimulus. In neurons where Ser133 phosphorylation is sustained, the CREB target gene, *c-fos*, is induced. By contrast, in neurons where Ser133 is transiently phosphorylated, *c-fos* is not induced (123). Duration of CREB phosphorylation in striatal neurons appears to be determined by the presence of a particular factor, dopamine and cAMP-regulated phosphoprotein (DARPP-32). Only DARPP-32–expressing cells exhibit prolonged CREB phosphorylation. DARPP-32 is activated when phosphorylated by PKA and, once phosphorylated, inhibits PP-1 (118). Thus, presence of DARPP-32 enables a cell to respond to cAMP by sustained CREB phosphorylation.

Cooperative Interactions with Other Transcription Factors

Under some circumstances, recruitment of CREB is sufficient to confer a cAMP-responsiveness to a heterologous promoter. In PC12 cells, a Gal4-CREB fusion protein can confer forskolin-responsiveness to a Gal4 reporter gene (65). However, in other cell types, CREB requires the presence (or removal) of additional promoter-bound factors to activate transcription. Although CREB is required for cAMP-induced activation of the phosphoenolpyruvate carboxykinase (PEPCK) gene (124), recruitment of CREB to the PEPCK promoter via a heterologous Gal4-CREB fusion is insufficient for PKA-activated transcription unless other upstream promoter elements are present, including binding sites for the bZIP factor C/EBP and the Fos-Jun activator protein 1 (AP-1) complex (124, 125). Interestingly, one study showed that recruitment of multiple Gal4-CREB molecules to the promoter bypassed the requirement for upstream cooperating factors, suggesting that these upstream factors may function to enhance or magnify an inherent activation function of CREB (124). However, in another study using a different cell line, recruitment of multiple Gal4-CREBs in the absence of upstream elements resulted in only modest or no transcriptional activation by cAMP (125). In this case, the findings imply that upstream factors cooperate with CREB to activate transcription through a mechanism that is somehow distinct from the way CREB activates alone.

Regulation of ATF-1 and CREM

Although CREM and ATF-1 share extensive similarity with CREB throughout the KID (Figure 6), including the PKA consensus site (RRPS), these two factors differ significantly from CREB in their ability to activate transcription in response to cAMP (32–34). When fused to Gal4 and tested for ability to activate a Gal4-driven reporter, ATF-1 was found to respond only weakly to cAMP. The minimal response that was detected required the presence of the leucine zipper domain, suggesting that in this context, Gal4-ATF-1 interacts with an endogenous bZIP protein, possibly CREB, to activate transcription (47, 126, 127). Although isoforms of CREM such as α, β, and γ contain a KID, they function not as activa-

tors, but as repressors of PKA-induced CREB and PKA-induced CRE transcription (34). Presumably, this situation arises because they lack the flanking constitutive activation domains (Q1 and Q2/CAD) present in CREB. Interestingly, phosphorylation by PKA within the CREM KID results in a slight decrease in CREM repression, suggesting a role for the KID in CREM function (35). In addition, just as the KID of CREB can cooperate with other transcription factor activation domains in *trans* to achieve high levels of stimulus-induced transcription, a repressor isoform of CREM can also be converted into an activator by recruiting another constitutive activation domain to the promoter. For example, co-expression of a Gal4-CREMα fusion with a LexA-Gcn4 fusion led to high levels of PKA inducibility of a reporter gene containing LexA and Gal4 binding sites within its promoter (76). In addition, CREMτ, which contains a KID and flanking activation domains homologous to Q1 and Q2/CAD, and is thus very similar to CREB, functions as a transcriptional activator in response to PKA (36).

Taken together, these results suggest that the KID, although necessary for PKA inducibility, is not sufficient and emphasize the importance of other structural domains in CREB and its other family members.

GROWTH FACTORS AND CREB

Identification of CREB as a Growth Factor-Inducible Transcription Factor

Initially, CREB was thought to be fairly selective in mediating transcriptional responses restricted to extracellular stimuli that elevate cAMP. In particular, CRE-driven reporter genes are unresponsive to growth factors that activate receptor-tyrosine kinases (128). However, use of anti-phospho-CREB antibodies revealed that CREB does become phosphorylated at Ser133 when cells are stimulated by a wide range of extracellular stimuli, including many growth factors (129, 130). This observation suggested that CREB might be involved in some way in growth factor-stimulated gene expression, even though a CRE alone was not sufficient to mediate growth factor induction. In the context of the *c-fos* promoter, mutation of the CREB-binding sites substantially reduced growth factor induction of *c-fos* transcription (129), indicating that CREB could contribute to the growth factor response. Additional work (57, 129) revealed that CREB plays a critical role in mediating growth factor induction of *c-fos* gene transcription, and showed that this induction occurs in a Ser133–dependent manner.

Growth Factor-Inducible CREB Kinases

Fractionation of nuclear extracts from nerve growth factor (NGF)-treated PC12 cells identified a 105-kDa NGF-stimulated protein kinase that phosphorylates CREB at Ser133 (129). Exhaustive purification and sequence analysis pinpointed the NGF-inducible kinase as RSK2, a member of the pp90 ribosomal S6 kinase

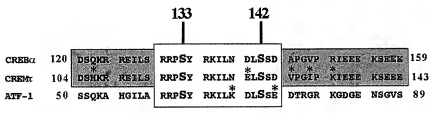


Figure 6 Comparison of the KID regions of CREB, cAMP response element modulator (CREM), and activating transcription factor 1 (ATF-1). Amino acid residues 120–159 of the KID of CREBα are shown in comparison with the homologous regions of CREMτ (104–143) and ATF-1 (50–89). The Ser133 and Ser142 of CREB are indicated, as well as the corresponding serines in CREMτ and ATF-1. The *yellow box* shows the high degree of identity between CREB, CREM, and ATF-1 in the region corresponding to amino acids 130–145 of CREBα. The *asterisks* within the yellow box indicate nonidentical residues. The *gray box* shows the homology between CREB and CREMτ in the region corresponding to amino acids 120–159 of CREBα, with *asterisks* indicating nonidentical residues.

(pp90^{RSK}) family of serine/threonine kinases (131). Although pp90 ^{RSK} was first detected in extracts from growth factor-treated cells as a kinase that catalyzed phosphorylation of ribosomal protein S6, further studies indicated that the pp90 ^{RSK} family does not phosphorylate S6 in cells (132). Rather, a primary function of the pp90 ^{RSK} family is to phosphorylate transcription factors such as CREB. NGF stimulation of target cells activates the NGF receptor, TrkA, which, via stimulation of guanine-nucleotide exchange factors (GEFs), such as Sos, activates the small G protein, Ras (Figure 7). Activated Ras interacts with and stimulates the Ser/Thr kinase, Raf, which triggers, sequentially, activation of the dual-specificity kinase, MEK, and its targets, the ERK1/2 members of the MAPK family of Ser/Thr kinases (133). One substrate of the ERKs is the ribosomal protein SG kinase of 90 kDa (RSK). On activation, both ERKs and RSKs translocate to the nucleus where RSKs can phosphorylate CREB at Ser133 (131, 134). Although RSK2 was identified as the NGF-inducible CREB kinase, further analysis revealed that all three RSK family members (RSK1–3) are activated by NGF, and that all can phosphorylate CREB at Ser133 in vitro and in vivo (135). Thus, all three RSKs likely contribute to growth factor-induced CREB phosphorylation. Whether a particular pp90 ^{RSK} mediates a growth factor response will depend on its level of expression. In addition to activating ERK and RSK, NGF treatment of PC12 cells also activates the p38 MAPK pathway (135). The p38 MAPK is activated by the upstream kinase, MKK6 (136), and catalyzes the phosphorylation and activation of two kinases, MAPKAP-K2 and MAPKAP-K3 (137, 138). MAPKAP-K2 was recently found to phosphorylate CREB, and through the use of pharmacological agents that selectively inhibit either the ERK (139) or p38 MAPK (140) path-

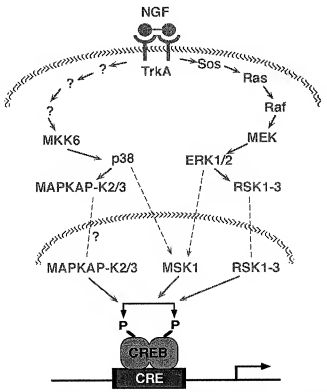


Figure 7 NGF-activatcd signaling pathways lcading to CREB Ser133 phosphorylation. Dimers of NGF bind to and activate the NGF receptor TrkA. Activated TrkA, via the Grb2/Shc adaptor complex (not shown) activates the guanine-exchangc factor Sos, which leads to thc subsequent activation of Ras and the downstream kinases Raf, MEK, and the MAPKs ERK1/2. Activated ERKs stimulate the activity of RSKs (RSK1-3), which then translocate to the nucleus and phosphorylate CREB at Ser133. ERKs can also translocate into the nucleus themselves to activate the kinase MSK1, which phosphorylates CREB at Ser133. Activated TrkA can also induce the activity of MKK6, a MEK-likc kinase which directly activates the p38 MAPK. The p38 can activate MSK1 as well as activate the kinascs MAPKAP-K2/3, and both MSK1 and MAPKAP-K2/3 can phosphorylate CREB at Ser133. The mechanisms by which TrkA activation leads to MKK6 activation are presently unclear. It is also not known whether MAPKAP-K2/3 are activated in the cytoplasm or in thc nucleus. *Dashed lines* indicate translocation from the cytoplasm to the nuclcus.

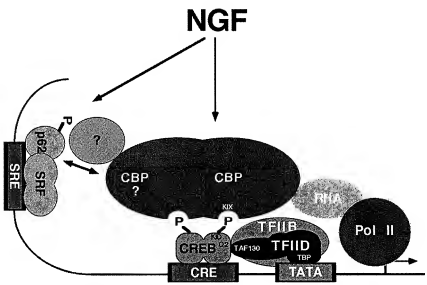


Figure 8 cAMP response element-binding protein (CREB) cooperates with other factors in activating transcription in response to NGF. NGF induces CREB Ser133 phosphorylation, which functions to recruit coactivator molecules such as CBP (see Figure 4). However, NGF activation of *c-fos* transcription requires promoter elements in addition to the CRE. Specifically, factors bound to the upstream SRE element are required for robust *c-fos* transcription. CREB may cooperate with a transcription factor complex bound to the SRE. This complex is composed of an SRF dimer and the associated factor $p62^{TCF}$ (p62), which becomes newly phosphorylated in response to NGF stimulation. Phosphorylated $p62^{TCF}$ and/or SRF may directly associate with proteins such as CBP to stabilize these coactivators on the promoter. It is also possible that SRE-bound factors such as $p62^{TCF}$ and SRF might interact with an as yet unidentified factor(s) (*light-blue circle with question mark*) to stabilize CBP on the promoter.

ways, it was shown that both of these pathways (via RSKs and MAPKAP-Ks) contribute to NGF activation of CREB phosphorylation (Figure 8; 135). MAPKAP-K2 has also been shown to mediate fibroblast growth factor (FGF) induction of CREB phosphorylation (141). Yet another new relative of $pp90^{RSK}$, termed MSK1, which is activated by both the ERKs and p38 MAPK, appears to also be a CREB kinase (142). Although it seems that many kinases can potentially phosphorylate CREB at Ser133 in growth factor-stimulated PC12 cells, evidence strongly implicates RSK2 as the primary CREB kinase in EGF-stimulated fibroblasts. In fibroblasts derived from patients with Coffin-Lowry syndrome, which lack RSK2 activity due to a mutation in the *RSK2* gene (143), EGF-induced CREB phosphorylation and *c-fos* expression are greatly diminished (144).

CREB Cooperates with Other Factors to Mediate a Response to Growth Factors

A unique feature of CREB involvement in growth factor signaling is that, under most circumstances, CREB is incapable of conferring a response in the absence of cooperating factors. The most well-studied growth-factor-inductible locus is the immediate-early gene (IEG), *c-fos* (1). In PC12 cells, mutating the serum response element (SRE) in the *c-fos* promoter (but leaving the CREB binding sites intact) abolishes growth factor induction (57). To activate *c-fos* transcription in response to growth factors in PC12 cells, CREB requires that serum response factor (SRF) be bound to the SRE (57). Because CREB becomes phosphorylated at Ser133 in growth factor-simulated cells, phosphorylation at Ser133 is not sufficient for CREB activation in the absence of CREB interaction with other promoter-bound factors (129). Thus, at certain promoters, CRE-bound factors must cooperate with SRE-bound factors to activate transcription in response to growth factor signals (Figure 8). Other transcription factors that mediate growth factor responses at SREs, such as SRF or $p62^{TCF}$ (another SRE-binding protein), may be required for efficient recruitment of CBP to the promoter (Figure 8). Consistent with this possibility, CBP interacts with $p62^{TCF}$ family members in vitro and enhances their ability to activate transcription in vivo (145). Under some circumstances, CREB may be capable of conferring a robust growth factor response in the absence of cooperating factors. In primary cortical neurons, the growth factor brain-derived neurotrophic factor (BDNF) stimulates both Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV) (Figure 9) and the Ras-MAPK cascade, leading to CREB phosphorylation at Ser133 and activation of CREB-dependent transcription (57, 146).

Growth Factor Inhibition of CREB-Mediated Transcription

Why might CREB under certain circumstances require the presence of other promoter-bound transcription factors? Perhaps certain signals initiated by growth factor stimulation interfere with CREB-CBP association. One study suggests that NGF stimulation of the Ras-MAPK pathway in PC12 cells induces a CBP-RSK interaction, thereby sequestering CBP and inhibiting CRE- and CREB-mediated transcription (147). In H4ie cells, insulin also represses cAMP-induced activation of CREB. Both NGF and insulin suppress CREB activity without reducing Ser133 phosphorylation (125, 147, 148). Studies of the PEPCK gene indicate that although the Ras-MAPK cascade can partially inhibit CREB-mediated transcription, the repressing effect of insulin is largely independent of Ras and occurs instead via a PI3-kinase–dependent pathway (149, 150). Detailed analysis of insulin-responsive PEPCK promoter elements suggests that insulin can inhibit both basal and PKA-induced transcription mediated by CREB. Insulin inhibits PKA-activated CREB most effectively when the CRE is in the context of other PEPCK promoter elements (125), implying that inhibition may occur at the level of coactivators that interact with both CREB and other promoter-bound factors.

Dependence of Growth Factor Signaling on Intracellular Ca^{2+}

Although the Ras-MAPK pathway is important for growth factor induction of CREB Scr133 phosphorylation, Ca^{2+}-signaling pathways also mediate CREB phosphorylation in some growth factor-treated cells. Exposure of cortical neurons to BDNF leads to a rise in intracellular Ca^{2+}, and inhibition of CaMK activity with pharmacological agents or dominant-interfering mutants partially blocked both BDNF-induced CREB phosphorylation and CREB-dependent transcription (146). In oligodendrocyte progenitor cells, basic fibroblast growth factor induced activation of MAPK and CREB Scr133 phosphorylation. However, activation of MAPK and CREB phosphorylation were completely blocked by chelation of intracellular Ca^{2+} (151).

Ca^{2+} AND CREB

Like cAMP, Ca^{2+} functions as a second messenger in a variety of cellular processes (152). In the nervous system, changes in membrane potential can increase intracellular Ca^{2+} levels via several mechanisms. Release of neurotransmitter at a synapse activates ligand-gated ion channels, such as the *N*-methyl D-aspartate (NMDA) receptor, a major glutamate receptor subtype which permits Ca^{2+} entry. Changes in membrane depolarization can open voltage-gated Ca^{2+} channels (VSCCs), stimulating influx of extracellular Ca^{2+}. Other neurotransmitter receptors, including muscarinic acetycholine receptors, are G-protein-coupled receptors that activate enzymes that provoke a rise in intracellular Ca^{2+} by stimulating release of Ca^{2+} from internal stores. Artificial membrane depolarization by application of high exogenous potassium chloride induces expression of IEGs in PC12 cells (153, 154). Characterization of the mechanisms by which Ca^{2-} influx through VSCCs activates *c-fos* transcription has provided insight into how Ca^{2+} induces gene expression

Identification of the CaRE as a Ca^{2+}-Responsive Element

Membrane depolarization of PC12 cells causes Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels (L-VSCCs) (153, 154). Ca^{2+} influx, in turn, activates *c-fos* transcription through a promoter element centered at -60 bp relative to the transcription start site (128, 153, 155). The -60 bp element of the human *c-fos* gene contains a sequence, TGACGTTT, similar to the 8-bp palindromic sequence of the somatostatin CRE (TGACGTCA). The -60-bp element binds a factor present in nuclear extracts (156) and binding is constitutive; binding activity is not affected by membrane depolarization and Ca^{2+} influx (155). The -60-bp Ca^{2+}-responsive element (CaRE) is also a cAMP-responsive element (157). This fact, together with the similarity between the *c-fos* CaRE and the somatostatin CRE, suggested that CREB or a CREB-like factor might be the target(s) of Ca^{2+}-activated signaling pathways. Several lines of evidence subse-

quently identified CREB as the CaRE-binding factor (158, 159): (*a*) CREB binds specifically to the CaRE sequence in vitro; (*b*) purification of the CaRE-binding activity in nuclear extracts revealed it to be CREB; (*c*) nucleotide mutations in the CaRE that disrupted transcriptional activation in membrane-depolarized cells also disrupted CREB binding; and (*d*) blockade of CREB function within cells inhibited membrane depolarization-induced CaRE-dependent transcription.

Membrane Depolarization Induces CREB Phosphorylation

Experiments show membrane depolarization and subsequent Ca^{2+} influx activate CREB by inducing CREB phosphorylation at Ser133 (159). In neurons, Ca^{2+} influx through NMDA receptors also causes phosphorylation of Ser133 (62). A Gal4-CREB fusion was capable of mediating membrane depolarization induction of a Gal4-dependent reporter gene, and mutation of Ser133 to alanine abolished Ca^{2+}-dependent transcription (65). The requirement for Ser133 phosphorylation suggested that CBP is also involved in membrane depolarization induction. Indeed, membrane depolarization induces interaction of CREB with CBP via the KID and KIX domain (A Bonni, JM Kornhauser, AJ Shaywitz, ME Greenberg, unpublished observations).

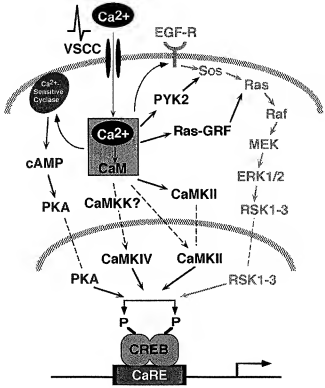
Given the relatedness of CREB family members within the KID, especially around Ser133 (or the equivalent) (Figure 6), it is not surprising that Ca^{2+} influx also results in phosphorylation of both ATF-1 (Ser-63) and CREM (Ser-117) (160, 161). Both ATF-1 and CREM stimulate transcription in response to Ca^{2+}; however, ATF-1 stimulates transcription to a greater extent in membrane-depolarized cells than in forskolin-treated cells (126, 161). By contrast, CREMτ induces transcription to a lower level in Ca^{2+} ionophore-treated cells than in forskolin-treated cells (160). Thus, other structural features of CREB, ATF-1, and CREM presumably account for these observed differences in function, but the mechanistic basis of these differences is not known.

Protein Kinases That Mediate the Ca^{2+} Response

Ca^{2+} is a pleiotropic second messenger that activates a variety of signal transduction pathways. Identification of the kinase(s) that catalyzes Ser133 phos-phorylation in membrane-depolarized cells has proved to be difficult. Many kinases, some of whose activities are enhanced by Ca^{2+}, are capable of phos- phorylating CREB in vitro, including Ca^{2+}/calmodulin-dependent kinases I, II, and IV (CaMKI, II, and IV), RSK1, RSK2, RSK3, PKC, and PKA. More thorough biochemical and cell biological analysis is necessary to determine which of these kinases mediates a Ca^{2+} response under a particular circumstance. However, an emerging view is that, in a given cell type, Ca^{2+} may activate several distinct signaling pathways that each culminate in CREB phosphorylation (Figure 9). The ability to achieve stoichiometric phosphorylation of CREB could possibly require that more than one CREB kinase be activated. In addition, CREB kinases activated with distinct kinetics could provide a mechanism to prolong response.

Ca^{2+}/Calmodulin-Dependent Kinases The best characterized of the Ca^{2+} activated CREB kinases are CaMKs. When intracellular Ca^{2+} concentration rises, the concentration of Ca^{2+}/calmodulin (CaM) complex increases. Ca^{2+}/CaM binds to and activates the CaMK family of serine/threonine kinases either directly, e.g. CaMKII, or indirectly via activating the upstream enzymes, e.g. CaMKI and CaMKIV (162, 163). The sequence containing Ser133 corresponds to the consensus for phosphorylation by CaMKs (RXXS). Phosphopeptide mapping indicates that CaMKI, CaMKII, and CaMKIV all phosphorylate CREB at Ser133 in vitro (65, 115, 164–166).

Involvement of CaMKs was suggested by the observation that inhibitors of CaM or CaMKs block membrane depolarization-induced *c-fos* transcription (154, 167, 168). Cumulative evidence indicates that a particular CaMK family member, CaMKIV, mediates membrane depolarization/Ca^{2+}-dependent activation of CREB. First, kinetics of CaMKIV activation within minutes after membrane depolarization correlate with stimulation of Ser133 phosphorylation (169). Second, CaMKIV is found in the nucleus, where CREB is localized (170). Third, cotransfection of constitutively active CaMKIV drives CREB- and CRE-dependent gene expression (115, 165, 166). Finally, interfering with CaMKIV



function, using either pharmacologic agents (such as the drug KN-93) that block activity or antisense oligonucleotides that block expression, inhibits membrane depolarization-induced Ser133 phosphorylation (169).

It is less likely that the other CaMK family members, CaMKI and CaMKII, activate CREB-dependent transcription in response to membrane depolarization. Although CaMKI phosphorylates CREB at Ser133 in vitro and can activate CREB-dependent transcription in transient transfection assays (128, 161), CaMKI does not appear to be localized to the nucleus in neurons (169, 171). CaMKII, on the other hand, does not activate CREB-/CRE-dependent transcription in transcription assays (115, 165, 166). This finding was unexpected, since in vitro CaMKII, when compared to CaMKIV, is a more potent activator of CREB Ser133 phosphorylation. Whereas both CaMKII and CaMKIV have similar affinities for CREB in vitro (K_m = 1 and 2.5 μM, respectively), the V_{max} for phosphorylation of CREB by CaMKIV is actually 30- to 40-fold lower than that by CaMKII (115).

Figure 9 Multiple signaling pathways contribute to cAMP response element-binding protein (CREB) Ser133 phosphorylation in response to Ca^{2+} influx. In neuronal cells, electrical activity leads to membrane depolarization, opening voltage-sensitive Ca^{2+} channels (VSCCs) in the plasma membranc and resulting in influx of extracellular Ca^{2+}. Insidc the cell, calcium activates many kinases, some of which directly phosphorylate CREB at Ser133. Upon entering the cell, Ca^{2+} binds to a protein, calmodulin (CaM). The Ca^{2+}/CaM complex (*shaded box*) can activate the PKA pathway (*blue*) by directly stimulating calcium-sensitive adenylyly cyclases, leading to generation of cAMP and the activation of PKA. PKA can then translocate to the nucleus where it phosphorylates CREB at Ser133. Ca^{2+}/CaM also activates members of the Ca^{2+}/calmodulin-dependent kinase (CaMK) family (*black*), all of which can phosphorylate CREB at Ser133. Ca^{2+}/CaM directly activates CaMKI (not shown), CaMKII, and CaMKIV. Ca^{2+}/CaM can also activatc CaMKK, which can then directly activate both CaMKIV and CaMKI (not shown). Nuclear translocation of Ca^{2+}/CaM may account for the activation of CaMKIV and CaMKII. CaMKIV is localized predominantly to the nucleus while isoforms of CaMKII are found both in the nucleus and in the cytoplasm (211). In addition, certain CaMKII isoforms may translocate from the cytoplasm to the nucleus (212). Ca^{2+}/CaM also activatcs the Ras/MAPK pathway (*red*). Ca^{2+} activation of Ras may occur through multiplc mechanisms. Ca^{2+} influx can lead to the ligand-indcpendent activation of the EGF-rceptor (EGF-R), which then leads to activation of guanine-nucleotide exchange factors, such as Sos and Ras activation. Activation of Ras stimulates the Raf, MEK, and ERK1/2 kinase cascade. The MAP kinases ERK1/2 directly activate members of the $pp90^{RSK}$ family of protein kinases (RSK1-3). Activated RSKs then translocate to the nucleus where they phosphorylate CREB at Ser133. Ca^{2+}/CaM can also activate Ras by activating Ras-GRF, a Ca^{2+}-activatcd guanine-nucleotide exchange factor. The calcium-activated tyrosine kinase PYK2 can also activate Sos and lead to stimulation of the Ras pathway. *Dashed lines* indicate translocation from the cytoplasm to the nucleus.

Mounting evidence indicates that CaMKII actually inhibits CREB activity by phosphorylating other sites on CREB in addition to Ser133 (see below).

Ca^{2+} and Ras CaMKs are not the only kinases that phosphorylate CREB at Ser133 in response to membrane depolarization. Under conditions in which CaMKIV is not expressed or is inhibited, membrane depolarization can still induce CREB Ser133 phosphorylation and lead to the activation of CREB-dependent transcription. For example, in PC12 cells, although there is no detectable CaMKIV expression (146), membrane depolarization still induces CREB Ser133 phosphorylation and activates CREB-dependent transcription (65). In AtT20 cells, the CaMK inhibitor, KN-62, effectively blocks membrane depolarization-induced CaMK activity (168), but does not block phosphorylation at Ser133 (172). One other pathway that may contribute to Ca^{2+} stimulation of Ser133 phosphorylation is the Ras/MAPK pathway. The Ras/MAPK pathway is a well-characterized mediator of the effects of growth factor receptor-tyrosine kinases (see above). However, it has been found that Ca^{2+} influx (via either NMDA receptors or L-VSCCs) can also trigger activation of the Ras/ MAPK cascade (173–175). In some cells, neurotransmitter stimulation evokes Ser133 phosphorylation through a pathway that is both Ca^{2+} and MAPK dependent (151).

How Ca^{2+} triggers Ras activation is at present only partly understood, but several mechanisms have been proposed (176). PYK2, a recently cloned tyrosine kinase, is activated by Ca^{2+} and is capable of translating a Ca^{2+} stimulus into Ras activation (177). PYK2 appears to function by recruiting GEFs that activate Ras to the membrane. How Ca^{2+} activates PYK2 is presently unknown, but a Src family tyrosine kinase may be involved (175). Another recently identified factor, Ras-GRF, also appears to mediate Ca^{2+} activation of Ras in neurons (178). Ras-GRF is a GEF that binds Ca^{2+}/calmodulin and activates Ras by catalyzing exchange of GDP for GTP. Finally, in PC12 cells, membrane depolarization induces EGF-independent tyrosine phosphorylation of EGF receptors that then recruit the Shc/Grb2/Sos complex and culminate in Ras activation (179).

Ca^{2+} and PKA In certain neuronal subtypes, Ca^{2+} influx leads to activation of PKA, which then phosphorylates CREB at Ser133. Ca^{2+} influx activates Ca^{2+}-sensitive adenylyl cyclase isoforms, increasing the level of cAMP, which activates PKA (180). This pathway may trigger Ser133 phosphorylation in AtT20 cells, where membrane depolarization stimulates CREB phosphorylation even when CaMK inhibitors are present (172).

Ca^{2+} Regulates CREB Activity Through Multiple Mechanisms

Kinetics of CREB Ser133 Phosphorylation Regulate CREB-Mediated Transcription Under certain conditions, Ser133 phosphorylation induced by membrane depolarization declines rapidly (within 30 min), whereas under other

circumstances phosphorylation is sustained for hours. As in the cAMP pathway, duration of Ser133 phosphorylation can affect the induction of gene expression in response to Ca^{2+}. For example, in hippocampal and striatal neurons, membrane depolarization induces expression of CRE-containing genes only if the stimulus produces prolonged phosphorylation (123, 169).

One way to control duration of Ca^{2+}-induced CREB phosphorylation is via regulation of the CREB phosphatases. The rate of CREB dephosphorylation could reflect either stimulus-induced activation of a phosphatase (with delayed kinetics relative to the kinase) or action of a constitutively active phosphatase as kinase activity declines. Ca^{2+} influx affects the activity of phosphatases that can act on CREB Ser133. The Ser/Thr phosphatase, calcineurin (CaN, also called PP-2B), is activated by Ca^{2+}/CaM (181) and can dephosphorylate Ser133-phosphate in vitro (115). As discussed above, PP-1 and PP-2A also dephosphorylate CREB at Ser133 in vitro and in vivo, and thus may mediate CREB dephosphorylation after membrane depolarization. Pharmacological studies indicate that PP-1 is the phosphatase that dephosphorylates Ser133 after membrane depolarization, at least in hippocampal neurons (169). However, CaN may also contribute because CaN can act indirectly to stimulate PP-1 activity (118). Indeed, in hippocampal neurons and striatal neurons, inhibition of CaN extends the duration of membrane depolarization-induced Ser133 phosphorylation (123, 169). Since certain patterns of membrane depolarization induce prolonged Ser133 phosphorylation, this suggests that a mechanism may exist to inhibit CaN activity under these conditions. Inactivation of CaN is also regulated by Ca^{2+}/CaM (182), so that neuronal stimuli that produce different patterns of Ca^{2+} influx may lead to either stimulation or inhibition of CaN activity. Other mechanisms have been invoked for regulation of CaN in response to different stimuli, including the generation of free radicals (183). How different types of Ca^{2+} stimuli lead to transient versus prolonged Ser133 phosphorylation is still unknown, however.

Ser133–Independent Regulation of CREB Activity Although the findings discussed above suggest that phosphorylation of CREB at Ser133 is a key event in membrane depolarization-induced CREB activation, modification of Ser133 alone is insufficient to explain the modulation of CREB activity by membrane depolarization. Membrane depolarization activates kinases that phosphorylate sites on CREB other than Ser133 that may function to activate or inhibit CREB-dependent transcription.

CaMKII phosphorylates CREB at Ser133 and at a second site (65, 164), Ser142, a residue that also lies within the KID (115, 165). Mutation of Ser142 to alanine (S142A) in a Gal4-CREB fusion permitted CaMKII-induced (165) expression of a Gal4-dependent reporter, suggesting Ser142 phosphorylation is normally inhibitory to CREB function. Consistent with this idea, CaMKII inhibition of CREB activity is not reversed by coexpression of constitutively active CaMKIV or PKA, both of which phosphorylate CREB at Ser133 but not Ser142 (165).

How the phosphorylation of Ser142 inhibits CREB activity is unknown. Most mutations at Ser142 either have no effect or actually increase transcription,

whereas a S142D mutation abolishes signal-induced activation of Gal4-CREB (82). The negatively charged Asp residue may mimic phosphorylation. Thus, electrostatic interactions at this site may be important for transcriptional inhibition, possibly by interfering with CBP binding because a negative charge at 142 might disrupt the hydrophobic interactions between KID and KIX (Figure 3) (79, 84, 184). However, S142D does not affect the KID/KIX interaction either in vitro or in vivo (82), but the analysis used the isolated KID and KIX domain rather than full-length CREB and CBP. Another study indicates that phosphorylation of Ser142 (and possibly Ser143) disrupts the KID/KIX interaction (184). Another mechanism by which phosphorylation of Ser142 may inhibit CREB activity is via recruitment of an as yet unidentified corepressor that interacts with Ser142–phosphorylated CREB.

The sequences surrounding and including Ser142 are well conserved in both ATF-1 and CREM (Figure 6), suggesting that phosphorylation of the corresponding serine in ATF-1 (Ser72) and CREMτ (Ser126) may also negatively affect the transcriptional activity of these factors. Phosphorylation at Ser72 in ATF-1 is currently a subject of controversy. One study concluded that ATF-1 functions analogously to CREB: In vitro, CaMKII phosphorylates ATF-1 at two sites (Ser63 and Ser72) and, in GH3 cells, Gal4-ATF-1 can be activated by coexpressed CaMKIV, but not by coexpressed CaMKII (161). However, others have found that CaMKII phosphorylates only a single serine on ATF-1 in vitro, and that ATF-1 is activated by CaMKII in F9 cells (185). Given the sequences of their KIDs, it would be surprising if CaMKII phosphorylated CREB, but not ATF-1, at the corresponding inhibitory site. High concentrations of CaMKII are required to phosphorylate CREM at Ser126 in vitro, yet ^{32}P-labeling experiments indicate that Ser126 (and Ser127) are both phosphorylated in vivo (160). Whether the phosphorylation of CREM at Ser126 and Ser127 affects CREM function has not yet been addressed.

CaMKs may regulate CREB activity by catalyzing phosphorylation at sites other than Ser133 and Ser142. In AtT20 cells, blocking CaMKIV activity inhibited membrane depolarization induction of Gal4-CREB-dependent gene expression, but did not inhibit Ser133 phosphorylation (172). These findings suggest that either CaMKIV is needed to phosphorylate another site on CREB that is required for activation or that CaMKIV phosphorylates another protein critical for Gal4-CREB-dependent transcription. CBP is a possible CaMKIV target because CaMKIV coexpression activates a Gal4-CBP fusion to induce transcription from a Gal4-dependent reporter gene (172). It is also possible that calcium influx may more directly modify proteins involved in transcription. For example, the newly identified protein DREAM binds to sequences within the *c-fos* gene to repress transcription in the absence of calcium (186a). However, when the level of calcium in the nucleus rises, DREAM forms a complex with calcium and releases from the DNA, resulting in transcriptional de-repression.

PKA is required for Ca^{2+} activation of CREB-dependent transcription, but is not required for Ser133 phosphorylation in response to membrane depolarization (58, 186). Similarly, in T cells, inhibition of PKA blocked stimulus-induced

CREB-mediated transcription, but did not block Ser133 phosphorylation (59). These findings indicate that a PKA-dependent event other than CREB Ser133 phosphorylation is required for Ca^{2+} induction of CaRE-dependent transcription. This second event may involve an effect of PKA on the KID/KIX interaction (59). Alternatively, PKA may phosphorylate and inactivate a repressor of CREB activity, or may stimulate components of the basal transcription machinery. Because it has been reported that PKA is required for the nuclear translocation of ERKs and RSKs (187, 188), the second PKA-dependent event may be mediated by these other kinases. The nature of the PKA-dependent event that controls Ca^{2+} induction of CaRE-dependent transcription is an important subject for future research.

Depolarization-Induced CREB Activation May Require CREB to Cooperate with Other Promoter-Bound Factors Experiments using a leucine zipper-deficient Gal4-CREB suggest that CREB may heterodimerize with another bZIP factor to activate transcription of a Gal4-dependent reporter gene in response to membrane depolarization (57). CREB cooperates with other promoter-bound factors to activate transcription of the *BDNF* gene in membrane depolarized cells (189, 190). In cortical neurons, membrane depolarization activates *BDNF* transcription (189, 191). Although the *BDNF* promoter contains a CRE, and CREB is required for its induction by membrane depolarization, robust activation of *BDNF* expression by Ca^{2+} influx is dependent on a promoter element located ~30 bp 5′ of the CRE, suggesting that neural-specific factor(s) that bind to this site may cooperate with CREB

CREB may activate *c-fos* transcription in membrane depolarized cells by cooperating with transcription factors bound 3′ to the site of initiation of transcription. Studies of *c-fos* transcription in PC12 cells show that although *c-fos* transcription is initiated to a certain degree in unstimulated cells there is a block to *c-fos* transcript elongation (58). The transcription elongation block site is located between *c-fos* exons one and two. Membrane depolarization/Ca^{2+} influx relieves the transcription elongation block, thereby allowing the synthesis of full-length *c-fos* RNA. Thus, membrane depolarization/Ca^{2+} influx may regulate a factor that binds at the site of the transcriptional elongation block and this factor may cooperate with CREB to activate transcription.

Spatial and Temporal Features of Ca^{2+} Signaling Affect CREB Transcription The route of Ca^{2+} entry plays an important role in determining which Ca^{2+}-dependent signaling pathways are activated (192). For example, activation of both L-VSCCs and NMDA receptors increases intracellular Ca^{2+}; however, specific features of each channel type and their subcellular localization will affect the signal transduction molecules that are activated. In hippocampal neurons, membrane depolarization-induced activation of L-VSCCs and glutamate-induced activation of NMDA receptors both lead to Ser133 phosphorylation (62). However, activation of NMDA receptors fails to induce CREB-dependent transcription, whereas stimulation of L-VSCCs induces robust activation of

CREB-dependent transcription (65, 159, 168). This differential effect depends, in part, on the duration of CREB phosphorylation (see above), since activation of NMDA receptors causes only transient Ser133 phosphorylation, but activation of L-VSCCs induces prolonged phosphorylation (SM Finkbeiner and ME Greenberg, unpublished observations).

NMDA receptors are generally located at post-synaptic dendritic sites many microns from the soma, while L-VSCCs are often localized to the proximal dendrites near the cell body. Therefore, as Ca^{2+} enters the neuron through each of these channels, it encounters different intracellular environments. The Ca^{2+} channels themselves may affect the intracellular environment encountered by incoming Ca^{2+} ions because the cytoplasmic domains of Ca^{2+} channels reportedly bind specific signal transduction molecules that could contribute to propagation of the Ca^{2+} signal (193). For L-VSCCs, experiments suggest that, as Ca^{2+} enters a neuron, it does not diffuse very far from its site of entry and must activate a signaling pathway close to the membrane (186). Potential signaling molecules could be components of the Ras pathway, a Ca^{2+}-sensitive adenylyl cyclase, or possibly CaM. Ca^{2+} influx through L-VSCCs induces translocation of CaM from the cytoplasm to the nucleus (194), where it might activate nuclear CaMKs. Ca^{2+} influx through L-VSCCs also activates the Ras/MAPK pathway leading to RSK induction. Two studies suggest that although CaMKs may be important for initial activation of CREB, RSKs appear to be important for phosphorylating Ser133 at later time points (172, 188).

The Ser133 phosphorylation is initiated by Ca^{2+} acting near its site of entry and does not require an increase in nuclear Ca^{2+} (186). However, nuclear Ca^{2+} may be critical for activating other events that are necessary for CREB-dependent transcription. Studies using Ca^{2+} chelators localized to the nucleus demonstrated that nuclear Ca^{2+} regulates CREB-dependent transcription in a Ser133-independent manner (172, 195).

Like its route of entry, the temporal pattern of Ca^{2+} influx can be a critical determinant of the signal transduction molecules that are activated (196–198). In dorsal root ganglion neurons, varying the frequency of Ca^{2+} spikes alters the extent and duration of *c-fos* induction (197). In these experiments, CREB phosphorylation at Ser133 was not strictly correlated with *c-fos* induction. Certain frequencies of Ca^{2+} spikes were found to induce Ser133 phosphorylation, but not *c-fos* activation. This observation could be explained by the existence of a second Ca^{2+}-dependent regulatory event (in addition to Ser133 phosphorylation) required for CREB-dependent transcription that is induced only by certain Ca^{2+} spike frequencies.

Target Genes–BDNF

A variety of regulatory pathways have evolved that control the timing and the nature of the CREB response to Ca^{+} influx. The precise control of CREB function is likely to be important both during development as well as in the mature nervous system. During mammalian development, neuronal activity and possibly

Ca^{2+} influx play an important role in the development of synaptic connections, raising the possibility that CREB is involved in this process (191, 199, 200). Supporting this idea is the finding that in embryonic cortical neurons, neuronal activity induces expression of BDNF by a CREB-dependent mechanism (189, 190). BDNF, in turn, is known to play a critical role in nervous system development (201). Several studies have also shown that BDNF acts at synapses to modulate synaptic transmission (202, 203). The finding that Ca^{2+} regulates BDNF transcription by a CREB-dependent mechanism, taken together with the observation that BDNF modulates synaptic function, provides a possible explanation for how CREB regulates synaptic function. However, BDNF is probably only one of a number of CREB target genes that regulate synaptic function. Identification of additional CREB targets should provide further insight into how CREB contributes to development and to adaptive neuronal responses.

OTHER KINASES THAT PHOSPHORYLATE THE KINASE-INDUCIBLE DOMAIN OF CREB

In addition to PKA, CaMKs, and RSKs, other kinases may contribute to the regulation of CREB activity by phosphorylating residues within the KID. Specifically, PKC, glycogen synthase kinase III (GSK-3), and casein kinase II (CKII) can all phosphorylate residues in the KID, and may affect CREB function. PKC can phosphorylate CREB in vitro at multiple sites, including Ser133, and phosphorylation by PKC has been suggested to affect CREB dimerization (41). In lymphocytes, stimulation of the B-cell surface immunoglobulin leads to CREB phosphorylation via a PKC-dependent pathway, but the direct CREB kinase in this case is unknown (204). PKC can activate MAPK, so it is possible that a MAPK-dependent kinase such as one of the RSKs may be the culprit (205). Indeed, T-cell receptor stimulation induces CREB phosphorylation through a PKC-dependent pathway that also requires MAPK activation (206). Moreover, functional RSK2 was required for T-cell receptor–induced CREB phosphorylation, raising the possibility that the PKC-MAPK-RSK-CREB pathway functions in B cells as well.

GSK-3 is a hierarchical serine/threonine kinase, meaning that it phosphorylates its target substrate only after the substrate has been phosphorylated by another kinase (207). A GSK-3 site at Ser129 is generated after prior phosphorylation of CREB Ser133 by another kinase such as PKA, and phosphorylation of CREB Ser133 is required for in vitro phosphorylation of CREB Ser129 by GSK-3 (208). PKA activation of CREB-dependent transcription is enhanced by GSK-3 and reduced by mutation of CREB Ser129 to alanine, suggesting that GSK-3 phosphorylation of Ser129 contributes to CREB activity, but, there is no evidence yet that Ser129 is phosphorylated in vivo.

Like PKC, CKII can phosphorylate CREB in vitro at multiple sites (70). One of these sites, Ser156, is also phosphorylated in vivo after cAMP stimulation of PC12

cells (79, 114). One study suggests that CKII also phosphorylates CREB at Ser142 and/or Ser143 (184). Since phosphorylation of CREB at Ser142 inhibits CREB-mediated transcription (see above), CKII may be a negative regulator of CREB.

Because of the similarity between CREB and CREMτ within the KID, these same kinases (PKC, GSK-3, and CKII) are predicted to phosphorylate CREMτ. Biochemical analysis indicates that many of the potential phosphorylation sites within the KID of CREB are also phosphorylated within the KID of CREMτ both in vitro and in vivo (209). The notable exception is phosphorylation of the GSK-3 site, which is not observed in CREMτ in vivo. The pp70 S6 kinase ($pp70^{S6k}$) has been reported to phosphorylate CREMτ at Ser117 (analogous to Ser133 in CREB) (210); however, phosphorylation of CREB by $pp70^{S6k}$ has not been reported and pharmacological evidence indicates that this kinase is not required for growth factor-induced CREB phosphorylation (129).

CONCLUSIONS

Understanding of the mechanisms by which extracellular stimuli induce changes in gene expression is critical for understanding how cells can adapt to environmental cues. CREB is a transcription factor that is the target of a variety of signaling pathways mediating cell responses to extracellular stimuli. CREB becomes phosphorylated on a critical residue, Ser133, in response to signals as diverse as peptide hormone stimulation, growth factor stimulation, and neuronal activity. The identification of CBP and the subsequent characterization of its interaction with Ser133-phosphorylated CREB has provided a basis for understanding how CREB activates transcription in response to these extracellular stimuli. However, it is becoming clear that regulation of CREB activity is complex and that this regulation involves more than just phosphorylation at Ser133. Other phosphorylation events on CREB and other promoter-bound factors appear to regulate the ability of CREB to induce expression of specific target genes in response to particular stimuli. The ability of CREB to be phosphorylated on Ser133 by many signaling pathways allows CREB to function as a sensitive barometer of environmental change; at the same time, the requirement for other phosphorylation events and other factors may allow CREB to adjust specific cellular responses to meet specific stimuli. One challenge for the future is to elucidate additional mechanisms within the CREB pathway that allow CREB to function with both sensitivity and specificity.

Another issue that needs to be addressed is the role of other CREB family members in regulating Ca/CRE-dependent transcription. Which stimulus-induced Ca/CRE transcriptional events require CREB itself, and which can be carried out by ATF-1 and CREM? In nonredundant cases, why is one factor utilized in preference to another? Here, too, a more detailed and thorough understanding of the mechanisms by which CREB activates transcription will be critical for understanding why there are differences in function between the CREB family members.

Finally, understanding of the physiologic significance of stimulus-induced CREB activation will require analysis of many more target genes induced by CREB. Mutation of a major growth factor-induced CREB kinase has deleterious effects on human development, as evidenced in Coffin-Lowry syndrome. Is this the result of a failure to activate particular CREB target genes? If so, what are these target genes? Identification of CREB target genes also will be important for understanding the molecular mechanisms of adaptive responses in the nervous system. How do stimuli that require activation of CREB within the nucleus of a single neuron result in the modification within the neuron of some synapses but not others? In addition to *BDNF*, other genes most likely exist that require CREB and are important for neuronal activity-dependent processes. Identification of these target genes and characterization of their function within the cell may provide insight into how signals that activate a nuclear transcription factor such as CREB achieve specificity at a subcellular level.

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CONTENTS

EXHIBIT A

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Education and profession:

1970	PhD Department of Science, University of Louvain, Belgium
1973	Postdoc, Rockefeller University, NY, USA
1974	Postdoc, UCSD, San Diego, USA
1974	Professor University of Namur, Belgium
1999	Founder of the spin off company "Advanced Array Technology"
2002	Director R-D Eppendorf Array Technologies (EAT)

Research Interests: Development of new DNA and protein biochips platform for genomic and for gene expression. Use of protein chips in complementation to the proteomic analysis by 2-D gel and mass spectra analysis. Applications for research as well as for diagnostic applications such as cancer prognostic and bacterial identification. Numerous collaborations with many research laboratories. Development and production of 8 biochips of gene expression analysis and genomic chips for the detection of bacteria.

Professional Experience:
Joined the University of Namur in 1974 and starting the laboratory for biochemistry and cell biology. Head of the laboratory since 1974.
Starting the research unit in cell biology (URBC) in 1997. The URBC is now composed of 65 researchers and technicians.
Member of many national and international committees, EEC expert in biotechnology programs.
R-D Director of Eppendorf Array Technologies, S.A.

Publications
The author's scientific output consists of 351 research papers in peer-reviewed international journals and inventor of 47 filed patents

5 Publications 5 last years

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